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# Usefulness of the Self-Fertilizing Cyprinodontid Fish, 'Rivulus marmoratus' as an Experimental Animal in Studies Involving Carcinogenesis, Teratogenesis and Mutagenesis

Charleston Coll., SC

Prepared for

Environmental Research Lab.
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# USEFULNESS OF THE SELF-FERTILIZING CYPRINODONTID FISH, RIVULUS MARMORATUS AS AN EXPERIMENTAL ANIMAL IN STUDIES INVOLVING CARCINOGENESIS, TERATOGENESIS AND MUTAGENESIS

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# FOREWORD

The protection of our estuarine and coastal areas from damage caused by toxic organic pollutants required that regulations restricting the introduction of these compounds into the environment be formulated on a sound scientific basis. Accurate information describing dose-response relationships for organisms and ecosystems under varying conditions is required. The EPA Environmental Research Laboratory, Gulf Breeze (ERL,GB), contributes to this information through research programs aimed at determining:

> the effects of toxic organic pollutants on individual species and communities of organisms;
>
> the effects of toxic organics on ecosystem processes and components;
>
> the significance of chemical carcinogens in the estuarine and marine environment.

Use of fishes as models for carcinogenic responses is an important step in the continued assessment of environmental contaminants. In this study are important data on a fish species particularly suited for laboratory screening of known and suspected agents. Both methodology for culture of the fish and responses to selected agents are reported. This information is presently being considered in on-going governmental programs dealing with broader questions of determination and evaluation of significance of agents in aquatic ecosystems.

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# ABSTRACT

*Rivulus marmoratus* is a naturally self-fertilizing cyprinodontid fish inhabiting mangrove marshes throughout the Caribbean. As a result of internal self-fertilization this oviparous species is composed of a number of isogenic, homozygous lines (clones), several of which have been identified by histocompatibility experiments and maintained in laboratory culture for over 30 years.

Simplified culture and handling methods are given and data are presented on the reproduction, growth and development of rivulus under laboratory culture as a prelude to the evaluation of its potential as a bioassay animal. Several types of bioassays were run and evaluated using rivulus: behavioral, carcinogenicity, teratogenicity, toxicity, and mutagenicity. Advantages and disadvantages of using rivulus for such bioassays are discussed. Behaviorally, rivulus is capable of detecting and avoiding water contaminated with $H_2S$. They respond (EC50 = 123.6 ppb $H_2S$) by leaping from the water and remaining emergent for various periods of time while respiring cutaneously. Hepatocellular carcinoma among other pathologic changes were observed in livers of rivulus a year after exposure of adults and larvae to diethylnitrosamine (45, 30 and 15 ppm in water) for 5 weeks and 12 weeks, respectively. No pathologic changes were found in embryos exposed similarly. High rates of various skeletal malformations resulted in offspring of adults exposed to dibutyl phthalate (DBP) and 2,3,4,6-tetrachlorophenol (TECP) at concentrations of 20, 10 and 5% (DBP - 0.740, 0.370, 0.185 mg/ℓ; TECP - 0.220, 0.110, 0.055 mg/ℓ) of the larval 96-hr LC50. No dose-response relationships of skeletal malformations were found for similar exposures to pentachlorophenol, 2,3,5-trichlorophenol or bromoform. However, chronic exposure of developing hatchlings to TECP resulted in fin and gill erosion and chronic exposure to bromoform produced dorsal fin abnormalities. As part of a mutagenesis bioassay 14 enzyme systems representing 28 loci were screened for the three laboratory clones and one wild-caught clone but no electrophoretic differences were found. Attempts to culture rivulus cells failed. Also, the karyotype of rivulus is not suitable for short-term cytogenetic assays such as the sister chromatid exchange (SCE) assay. Alternatively, however, the toadfish (*Opsanus tau*) possesses a suitable karyotype for SCE analysis and we have been successful in culturing toadfish cells to fourth passage. Increased rates of SCE were obtained when toadfish cells were exposed *in vitro* to the mutagen ethyl methanesulfonate but not bromoform. Another set of experiments is presented which involves characterization of the nature of the toadfish cytochrome P450 system.

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## SECTION 1

## GENERAL INTRODUCTION

The concept of a relationship between cancer and environmental contaminants has become prominent in the minds of many medical and environmental researchers. The enormous array of synthetic organic chemicals in the environment, many of which are quite persistent, and are expected to or have been shown to bioaccumulate, constitute a potential environmental hazard. The assessment of this potential hazard is, however, very difficult. A conference sponsored by the New York Academy of Sciences (Kraybill et al., 1977) relating pollutants and neoplasia in aquatic animals pointed out the considerable confusion that exists, not only in the assessment of the potential hazards but even in defining the potential effects.

It is important to recognize that the term cancer encompasses a group of diseases caused by a multitude of intrinsic and extrinsic factors acting singly or in combination. Carcinogenesis was defined by Miller (1970) as an heritable and at least quasipermanent loss of control of cell multiplication. Several mechanisms have been suggested to account for such a change in cell behavior: 1) interaction of carcinogens with DNA resulting in genic or chromosomal mutation, 2) alteration of specific protein or RNA to produce heritable changes in genome expression, 3) the activation of latent carcinogenic virus genome and 4) the selection of preneoplastic cells by conditions that favor the multiplication and survival of these cells. The latter two probably constitute indirect mechanisms operating through the first two (Fishbein, 1975).

The assessment of genetic damage then, is an important aspect of the assessment of potential carcinogenicity. Indeed, a number of screening tests for chemical carcinogens are dependent upon the correlation between carcinogenicity and mutagenicity. Fishbein (1975) states that it appears that many, if not all, chemical carcinogens are potential mutagens and probably most mutagens are potential carcinogens. This suggestion is credible because carcinogenesis is thought to be a multistep process with genetic alteration as the initial step. The relatively long induction periods for carcinogenesis may then be dependent upon the remaining steps in this process which in turn are related to a number of extrinisic and intrinsic factors.

Alterations in the genetic material may also cause teratogenic effects which could be mediated either by way of the parental adults or as a direct effect on the developing embryos. Although teratogenesis may be induced by many agents other than chemical mutagens (e.g., physical trauma, dietary deficiency, etc.) the relationship to mutagenesis and therefore carcinogenesis is evident.

Teleostean fishes show great promise as experimental animals in mutation research (Schroder, 1973). Their potential value in cancer research was clearly brought out at the conference on "Aquatic Pollutants and Biological Effects with Emphasis on Neoplasia" (Kraybill *et al.*, 1977) and the "Symposium on the Use of Small Fish Species in Carcinogenicity Testing" (sponsored by NCI and EPA, December, 1981). A number of papers given at these conferences also pointed out the high incidence of neoplasia in certain marine and freshwater fishes associated with areas of high industrial pollution. The consensus was the some fishes appear quite sensitive to tumorogenic agents and that an urgent need, voiced by Dr. M. F. Stanton (National Cancer Institute, Bethesda, Maryland), exists for a species "supersensitive to carcinogens."

Such a need is readily recognized and a possible candidate, which is the focus of this report, is the oviparous cyprinodontid fish, *Rivulus marmoratus*. It is the only fish known that exhibits natural syncronous hermaphroditism with internal self-fertilization (Harrington, 1971). This ultimate mode of inbreeding apparently has rendered these fishes homozygous and three isogenic clones (designated NA, DS, and M) have been identified by intraclonal, interclonal and interclonal hybrid offspring tissue transplants (Kallman and Harrington, 1964; Harrington and Kallman, 1968). These clones were carefully maintained in the laboratory of Dr. Robert W. Harrington, Jr. for nearly 20 years until his untimely death in 1975. The majority of the biological information known about this remarkable species is due to the meticulous efforts of Dr. Harrington (Harrington and Rivas, 1958; Harrington, 1961; Harrington, 1963; Kallman and Harrington, 1964; Harrington, 1967; Harrington and Kallman, 1968; Harrington, 1968; 1971; Lindsey and Harrington, 1972; Harrington, 1975; Massaro and Harrington, 1975; Harrington and Crossman, 1976). A complete pedigree was kept for each individual fish in the collection.

*R. marmoratus* is small (20-50 mm at maturity), easily maintained in the laboratory, has wide tolerances of salinity and temperature and oviposits up to ten fertilized eggs per week (during daylight hours, modally at noon). Generation time is on the order of 4-6 months. Eggs are large (1.5-2.0 mm diam) with a transparent chorion (shell). Embryonic development is of sufficient length (12-15 days at $25^{\circ}$C) to make careful observations on developmental rate and sequence and embryonic stages are somewhat similar to those of other cyprinodont fishes, for example, *Fundulus heteroclitus* (Armstrong and Child, 1965) and *Adinia xenica* (Koenig and Livingston, 1976).

The natural habitat of *R. marmoratus* is shallow estuarine mangrove marshes. Although the species is widely distributed throughout the Caribbean, it is somewhat rare in the continental United States.

Males of *R. marmoratus* were extremely rare in Dr. Harrington's field collections and females were never found. Dr. Harrington, however, demonstrated that males could readily be induced in his laboratory strains.

Two types of males exist, primary males, in which ovarian development never takes place and secondary males, which develop from hermaphrodites (ovarian degeneration). Primary males may be produced in the laboratory by submitting the terminal stages of embryonic development to low temperatures. Secondary males may result from hermaphrodites spontaneously or from exposure of hermaphrodites to short day length.

It is obvious from the preceding discussion that *R. marmoratus* possesses numerous attributes that make it particularly attractive as an experimental animal for carinogenicity, teratogenicity and mutagenicity studies. Its hardiness, ease of maintenance, developmental aspects, short generation time, fecundity and unique genetic aspects coupled with the fact that it is an estuarine fish with wide salinity tolerances all serve to increase its attractiveness, especially in light of recent evidence of increased incidence of neoplasms and other pathologic changes in marine organisms which may reflect accelerated deterioration of coastal marine environments.

The purpose of this work is to investigate the potential of *R. marmoratus* as an experimental animal useful in standard bioassay studies and particularly in the study of carcinogenicity and teratogenicity. Related objectives include the establishment of simplified culture methods and basic biological data while fish are held under laboratory conditions.

## SECTION 2

## CONCLUSIONS AND RECOMMENDATIONS

Large numbers of rivulus (and all stages of the life cycle) may be held efficiently in the laboratory in stackable glass culture dishes. Three feedings of brine shrimp per week and monthly water changes are sufficient to ensure health. As with any laboratory animal success in maintaining a healthy colony lies with the culturist and is directly proportional to TLC. We recommend that raw seawater not be used in the culture of rivulus to reduce chances of pathogen introduction.

Embryonic development of rivulus is similar to that of other cyprinodontid fishes. However, early development occurs to varying degrees within the ovotestis. This could cause problems in a study in which observation of early development (blastulation) is required. Also, because eggs are not fertilized synchronously it is difficult to get eggs in the same stage of development from a small group of egg-layers. A healthy egg-layer produces about an egg a day; however, trauma may bring about extended refractory periods. Problems associated with extended delays in hatching can be averted by mechanically removing the chorion, chemically changing the incubation medium which is moderately successful in stimulating hatching.

Rivulus avoids dissolved $H_2S$ (EC50 = 123.50, ppb $H_2S$) by leaping from the water and remaining emergent for various periods of time while respiring cutaneously. Further development of a bioassay based on this quantal response is recommended as it could be useful in water quality management.

Hepatocellular carcinoma, cholangioma, adenofibrosis and granuloma were induced in the livers of rivulus one year after exposure as adults and larvae to diethylnitrosamine (DEN) in water concentrations of 45, 30, and 15 ppm for 5 weeks and 12 weeks, respectively. No pathologic changes were observed in embryos treated similarly. It is recommended that further studies be done on the susceptability of rivulus to various carcinogens. In the long-term, this should be extended to clones more divergent from those used in this report, laboratory produced or derived from wild-caught fish, from a number of areas throughout the Caribbean. Such research may produce another inroad into the relationship between genetics and cancer.

Exposure of reproducing rivulus to the teratogen dibutyl phthalate (DBP) in water concentrations of 0.740, 0.370 and 0.185 mg/$\ell$ (20, 10 and 5% of larval 96-hr LC50) induced a variety of skeletal abnormalities in a dose-response fashion. A similar but less dramatic effect was induced by 2,3,4,6-tetrachlorophenol (TECP) but not by pentachlorophenol (PCP), 2,3,5-trichlorophenol (TRCP) or bromoform. Although rivulus appears quite sensitive to agents which induce skeletal malformations, the control rate (30%) is quite high. Preliminary work suggests that other clones, derived from recent wild-caught fish have spontaneous rates much lower than this. Because no aquatic teratogenicity assay such as this exists we strongly recommend that it be further developed.

Chronic exposure of rivulus parental adults and offspring through adult stage to 2,3,4,6-tetrachlorophenol (TECP) resulted in gill and fin erosion in offspring which showed a dose-response relationship. Exposure levels were 20, 10, and 5% of the larval 96-hr LC50 (0.220, 0.110 and 0.055 mg/ℓ). It is not known whether a pathogen is secondarily involved; however, the mechanisms of fin and gill erosion may be studied using rivulus as a model as such effects have been noted in several estuaries in the United States.

Chronic exposure of rivulus parental adults and offspring through adult stage to bromoform resulted in abnormal dorsal fin development in offspring. Exposure levels were 20, 10, and 5% of the larval 96-hr LC50 (8.4, 4.2 and 2.1 mg/ℓ). Such a highly specific effect was not expected. Further research is recommended to corroborate these findings and if corroborated to study the mechanism.

Fourteen enzyme systems representing about 28 loci were screened. However, no electrophoretic differences were found among the three laboratory clones or between clones and wild-caught rivulus taken near Naples, Florida.

Rivulus was found unsuitable for sister chromatid exchange (SCE) assay because chromosomes are small and numerous. The toadfish (*Opsanus tau*), however, has a suitable karyotype and *in vitro* exposure of toadfish leukocytes to ethyl methanesulfonate, a mutagen, induced increased rates of SCE.

Culture of rivulus cells has not been successful; however, toadfish cells have been cultured up to fourth passage.

The effects of hepatic microsomal enzyme (S-9) preparations from rats and toadfish pretreated with enzyme inducers 3-methyl-cholanthrene (MC) and arochlor (AC) and untreated on the metabolism of benzo (a) pyrene (BP) and 2-aminoanthracene (AA) to *Salmonella* mutagens demonstrate the similarities between these two species in their ability to metabolize promutagenic xenobiotics.

The toadfish shows promise for these types of research and we recommend that studies continue. Maddock is presently pursuing this work under another grant.

# SECTION 3

## LABORATORY CULTURE OF *RIVULUS MARMORATUS*

The following culture methods have been found optimum for reproduction, development and growth of *R. marmoratus*. These methods apply throughout this report. Adults are held singly in 4.5 in (11.4 cm) diam stackable culture dishes filled with 200 ml of 14 o/oo synthetic seawater prepared by dissolving approximately 14 g of Instant Ocean$^{R}$ sea salts per liter of distilled water. Adults are fed frozen brine shrimp (*Artemia*) three times a week taking care not to overfeed. A mature adult should not be fed more than about 9 large brine shrimp per feeding and small adults about 3. All uneaten food should be removed within 1 hour after feeding. Culture dishes are washed and seawater is changed monthly. Under a daily feeding regime, typically used when maximum growth rates are desired, seawater should be changed twice a month.

Larvae and juveniles are held singly in 2 in (5 cm) diam stackable culture dishes filled with synthetic 14 o/oo seawater. Young fish less than 3 weeks old may be held in groups; however, older fish become quite aggressive often resulting in death for most members of a group. Larvae and juveniles are fed freshly hatched brine shrimp nauplii three times a week. To prepare the brine shrimp culture 2-6 ml of brine shrimp eggs (San Francisco Brand) are added to a liter of synthetic 14 o/oo seawater in a 1 liter glass separatory funnel outfitted with an air stone, hose and air pump for aeration. After 24 hours of incubation at 25 C the air stone is removed for several minutes. The nauplii fall to the bottom and the egg capsules rise to the surface. Nauplii may then be tapped off in concentrated form and fed directly to rivulus larvae and juveniles. If concentrated nauplii are allowed to stand most will die within twenty minutes due to oxygen depletion. However, live nauplii will remain alive in rivulus culture dishes for several days providing a constant supply of food. Rivulus larvae and juveniles should not be fed dead nauplii or nauplii from a culture much older than 24 hrs.

Juveniles are transferred to 11.4 cm diam culture dishes about 2 months after hatching at which time the diet may be changed from brine shrimp nauplii to frozen adult brine shrimp or both may be included in the diet (see section on "Growth").

Eggs are incubated in 5 cm diam stackable culture dishes filled to 1 cm depth with synthetic 14 o/oo seawater. During the final stage of embryonic development hatching must often be induced otherwise embryos may delay hatching for considerable periods of time which may result in the death of the embryo. Hatching can be induced by changing daily the incubation water to either freshly collected seawater or naturally stained freshwater (see "Embryonic Development" section for discussion of delayed hatching).

Eggs can be collected from hermaphrodites by holding them singly in specially designed reproduction chambers (Fig. 1). These consist of 11.4 cm culture dishes with Teflon$^R$ screens (3-4 mm openings) suspended off the bottom of each dish by a glass ring ( 1 cm thick, 10 cm diam). Teflon screens are tied to the glass rings with fine monofilament line which effectively confines the fish to the water space above the screen. Most eggs accumulate between the walls of the culture dish and the glass ring, thus preventing rivulus from eating its own eggs. Eggs and any feces or uneaten food should be pipetted from the reproduction chambers 2-3 times per week. This can be most efficiently done immediately after feeding. Fish in reproduction chambers are fed individually by dangling thawed brine shrimp just in front of them with fine tissue forceps. Six or more can be fed at a time by proceeding from one to another repetatively until the entire group is fed to satiation. The optimum temperature and photoperiod for reproduction as well as all other phases of the life cycle is 25 C and 14 hrs, respectively.

There are numerous advantages to this culture system for research purposes that are available from no other fish culture system, marine or fresh water. The system is inexpensive, easy to maintain, records may be kept for each individual fish and large stocks may be kept in a relatively small space. In addition, all culture materials are made of either Teflon or glass, which is essential to avoid contamination by such ubiquitous substances as phthalate ester plasticizers.

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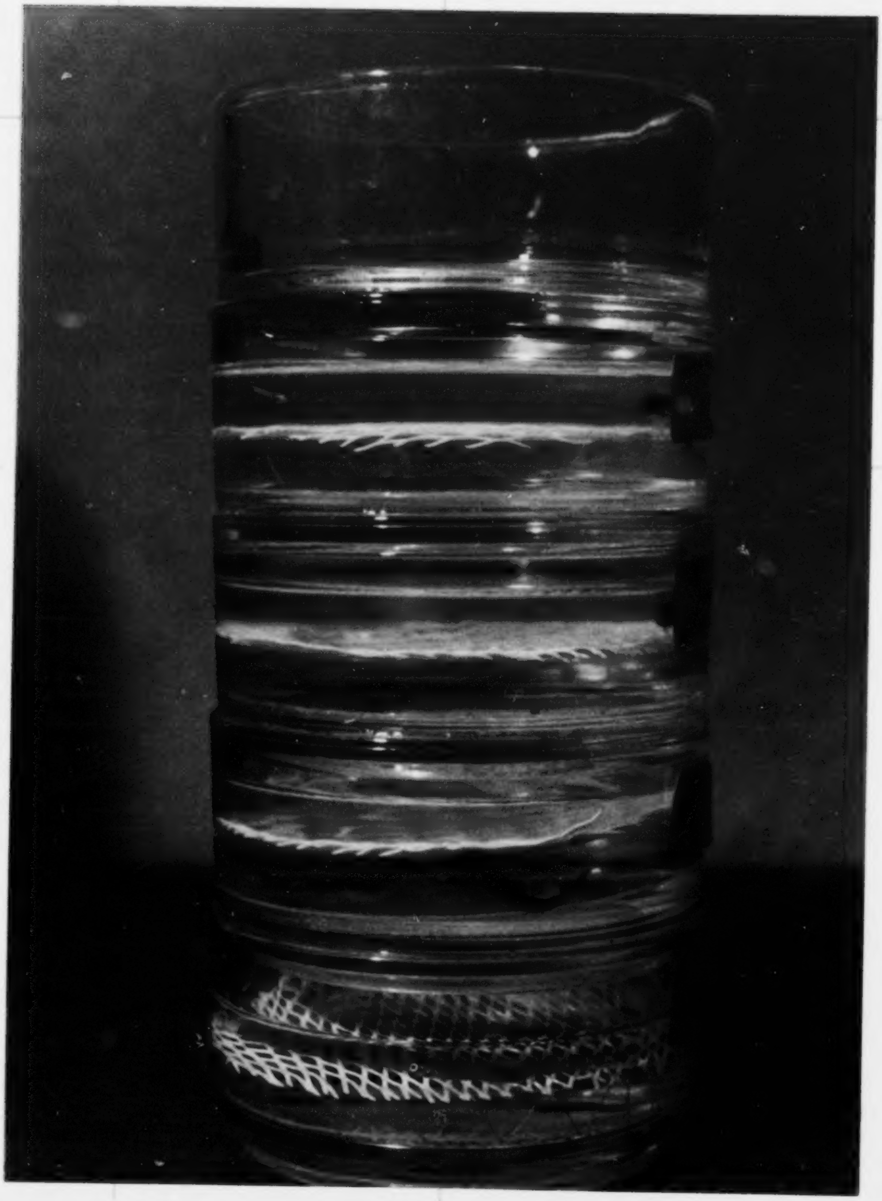

Figure 1. Photograph of Reproduction Chambers for Rivulus marmoratus.

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## SECTION 4

## REPRODUCTION, GROWTH AND DEVELOPMENT OF RIVULUS MARMORATUS UNDER LABORATORY CULTURE

### GENERAL OBSERVATIONS

The process of internal self-fertilization in R. marmoratus causes many unusual characteristics. The most readily noticed characteristic is that eggs are released in various stages of development. Eggs may be released from the parental fish singly or in clusters held together in a clump or long chain by sticky filaments (Fig. 2). Usually several eggs in a cluster are at different stages of development. The differences in the stages can range from a few hours to 24 to 48 hours. There is no particular sequence tothe release of eggs; eggs in an early stage may be released prior to eggs in a more mature stage. As Harrington (1963) noted in his observations, the eggs are probably released in random order after newly fertilized eggs become mixed with older developing eggs within the lumen of the ovotestis.

Why eggs are held within the parental fish for various periods of time and what triggers the release of one or several eggs are unknown. Harrington (1963), in his study on the sexual rhythms of rivulus, found that only 1% of the eggs observed were emitted within one hour of fertilization but that 80% were emitted within 24 hours of fertilization. The mean intraparental incubation time of all eggs observed was 10 hours. Therefore, eggs were usually retained within the body of the parental fish until the late blastula stage.

### FECUNDITY

To estimate fecundity of R. marmoratus held singly in reproduction chambers under the conditions described in the section "Laboratory Culture of Rivulus marmoratus" of this report, seven fish were observed over a four month period. These fish had been acclimated to the conditions in the reproduction chambers for four months (Nov.-Feb.) prior to the four months (Mar-June 1979) observation period. All were between 30-35 mm in length (standard length, SL), healthy proven egg layers. Three belonged to clone NA, two to clone DS and two to clone M.

Egg production for the seven fish averaged 25.3 eggs per month (SE = 2.27, N = 28). Clone NA fish averaged 26.8 eggs per month (SE = 3.9, N = 12), clone DS,20.5 eggs per month (SE = 3.8, N = 8) and clone M,

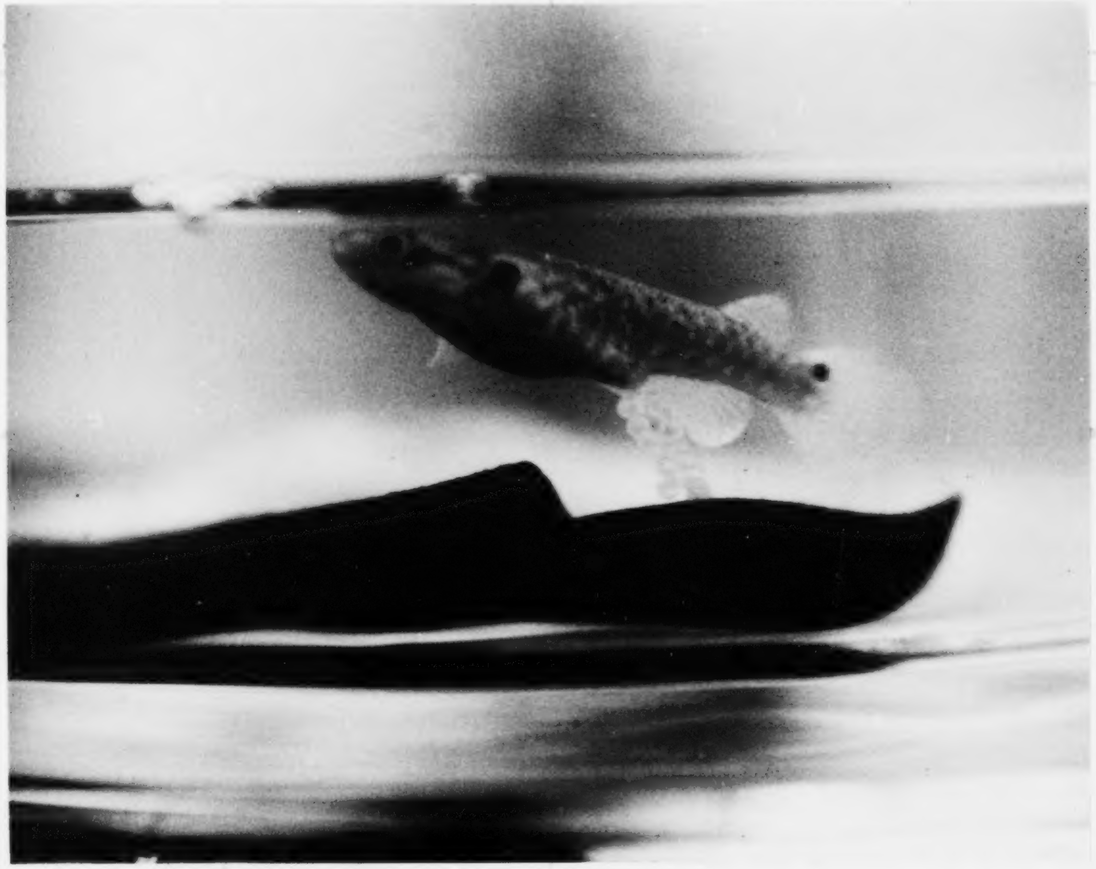

Figure 2. Photograph of Rivulus marmoratus with a cluster of eggs.

produced 27.9 eggs per month (SE = 3.8, N = 8). No statistical difference in egg production could be shown between clones (ANOVA; F = 0.9, p >.05). Overall 22.5% of the eggs laid were non-viable. Most of these were probably unfertilized although to make that statement with certainty necessitates collection of the eggs immediately upon oviposition (see Harrington, 1963). Harrington (1963) carefully observed 6 fish, 4 large (40-50 mm SL) and 2 small (25-30 mm SL) over a 4 month period (Aug-Nov). Eggs were collected immediately upon oviposition. Average egg production was 41.5 eggs per fish per month. However, the culture system, food and feeding regime were different as well as the average size of the fish. Our fish produced more eggs the longer they were held in the reproduction chambers. For example, the average egg production in March was 11.3 eggs per fish but the average production in June was 38.0 eggs per fish. The average egg production of Harrington's (1963) fish in August was 44.5 eggs per fish but in November was 30.0 eggs per fish. However, his fish were under a natural light regime. The effect of season on our fish, held under constant conditions is unknown. As a rule of thumb, a researcher working with medium sized, healthy adult rivulus using the culture system described in this report can expect about 1 egg per fish per day.

## GROWTH

To determine the growth rate of rivulus relative to container size, a rearing experiment was set up with three container sizes. All containers were stackable culture dishes and there were four replicates for each dish size. Dish diameters were 20.3, 11.4 and 5.1 cm and all were filled to a depth of 2 cm with synthetic 14 o/oo seawater. Uniform lighting was provided by 4-40 watt cool-white flourescent light bulbs suspended about 1 m above the fish. Standard 14 hr photoperiod and 25.0 ± 0.5 C were maintained throughout except for a 24 hr period in February when a power failure caused the temperature to drop to 17 C. Growth rate was recorded by periodically photographing each fish in its container next to a metric ruler. At the beginning of the study (4 Sept 1979) 12 larvae, all hatching on that day and all of the clone DS, were randomly assigned to the various containers. All dishes were washed and water changed weekly. All fish were fed equal amounts (in random order) of brine shrimp nauplii three times a week for the first 85 days. The quantity of nauplii was adjusted so that there were some remaining at each successive feeding. The young fish could thus feed *ad libitum*. All fish were switched from brine shrimp nauplii to frozen adult brine shrimp on the 86th day and they remained on this diet for the next 140 days at which time the study was ended. Frozen brine shrimp were also fed in random order with equal numbers of shrimp to each fish.

Summary data from the experiment are presented in Table 1 and the growth rate of fish in the 11.3 cm dishes is depicted in Figure 3. The growth rate was rapid during the first 2-3 weeks then became almost linear with time until about 160 days at which time it began leveling off.

Rate of growth was related to the size of dish (Table 1). The greatest difference in mean size among the dishes occurred 85 days after hatching and the effects of the smallest dish were greatest. It is possible that

Table 1. Growth of *Rivulus marmoratus* in Three Container Sizes

| Date | Day After Hatch | Diameter of Dish(cm) | TL(mm) | SE | N | Significance[a] |
|---|---|---|---|---|---|---|
| 4 Sept 1978 | 0 | 5.1 | 5 | 0 | 4 | NS[b] |
| | " | 11.4 | 5 | 0 | 4 | |
| | " | 20.3 | 5 | 0 | 4 | |
| 21 Sept 1978 | 17 | 5.1 | 9.0 | 0 | 4 | |
| | " | 11.4 | 9.5 | 0.29 | 4 | NS |
| | " | 20.3 | 10.1 | 0.43 | 4 | |
| 27 Oct 1978 | 53 | 5.1 | 10.8 | 0.25 | 4 | |
| | " | 11.4 | 12.3 | 0.63 | 4 | $p < 0.05$ |
| | " | 20.3 | 13.3 | 0.25 | 4 | |
| 28 Nov 1978 | 85 | 5.1 | 13.5 | 0.29 | 4 | |
| | " | 11.4 | 16.6 | 0.24 | 4 | $p < 0.001$ |
| | " | 20.3 | 17.3 | 0.25 | 4 | |
| 24 Jan 1979 | 142 | 5.1 | 21.3 | 0.33 | 3[c] | |
| | " | 11.4 | 22.3 | 0.25 | 4 | NS |
| | " | 20.3 | 23.7 | 1.2 | 3[d] | |
| 27 Mar 1979 | 204 | 5.1 | 25.0 | --- | 1[c] | |
| | " | 11.4 | 26.5 | 0.29 | 4 | NS |
| | " | 20.3 | 26.3 | 0.44 | 3 | |
| 17 Apr 1979 | 225 | 5.1 | 25.0 | --- | 1 | |
| | " | 11.4 | 26.8 | 0.25 | 4 | NS |
| | " | 20.3 | 26.7 | 0.33 | 3 | |

[a] Determined by single factor ANOVA

[b] NS = not significant = $p > 0.05$

[c] Numbers reduced by death of fish

[d] One fish jumped out of container and was lost

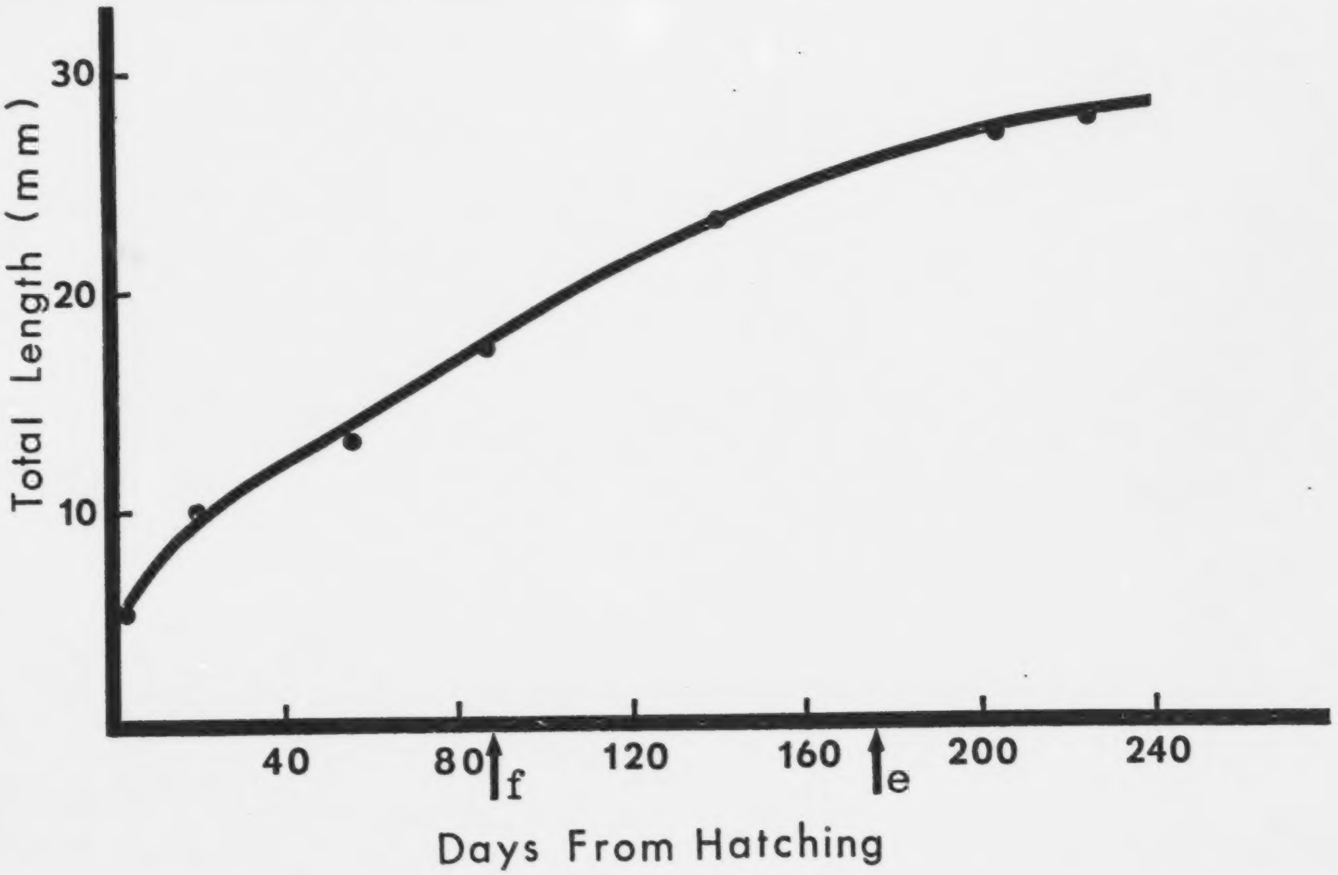

Figure 3. Growth of *Rivulus marmoratus* in the laboratory under controlled conditions: 11.4 cm diam culture dishes, 25.0 ± 0.5 C, 14 o/oo salinity and 14 hr photoperiod. Arrows represent time in which diet was changed from live brine shrimp nauplii to frozen adult brine shrimp (f) and time when first egg was laid (e).

feeding nauplii to older fish may have influenced the divergence in size. The diet was changed on the 86th day to frozen brine shrimp and all subsequent measurements were not significantly different among the dish sizes.

One fish jumped out of a large dish 28 Nov 1978 and was lost. All other mortalities occurred in the smallest dishes: 1 died 26 Dec 1978 (113 days after hatching), 2 died 26 Feb 1979 (175 days after hatching), and 1 died 17 Apr 1979 (225 days after hatching). The first egg was laid 28 Feb 1979 (177 days after hatching) and other fish followed during the subsequent weeks.

The optimum size culture dish is 11.4 cm diam because growth rate was nearly the same as in the largest dishes. Also, the 11.4 cm dish is much easier to handle and store. The smallest dish (5.1 cm) is clearly too small for full life cycle culture but is convenient for eggs, larvae and juveniles up to about 2 months old. Food type should also be switched from nauplii to frozen brine shrimp at about 2 months after hatching.

## EMBRYONIC DEVELOPMENT

This description of the embryonic stages of *Rivulus marmoratus* was patterned after Armstrong and Child's (1965) description of the embryonic development of cyprinodontid fish *Fundulus heteroclitus* and Koenig and Livingston's (1976) description of the embryonic development of the cyprinodontid fish *Adinia xenica*. Embryonic development is described as occurring in stages. The term stage refers to a discrete event or series of events in the developmental sequence. In this study, each stage is centered around an easily recognized event and also includes various minor changes which occur during the same time period. All stages are observable with the aid of a dissecting microscope. All times given are midpoints of stages.

Eggs used for determination of stages were obtained after they were naturally emitted from the parental fish. These eggs were collected at least once a day. A minimum of 15 eggs were observed for determination of each stage.

Eggs were handled with flexible forceps or eye droppers and observed on depression microscope slides or in 5.1 cm diam culture dishes. For ease in observation the later stages were dechorionated using stainless steel watchmaker forceps.

Observations were made using a Wild M-5 binocular microscope at 25 X magnification with transmitted and/or reflected light. All photographs of developmental stages were taken at 25 X or 10 X magnification with a Nikon camera attached to the microscope.

The following description of the stages of development of *R. marmoratus* under the controlled conditions of 25.0 ± 0.5 C, 14.0 ± 0.5 o/oo salinity, and 14 hours of light per day. The stages include development from fertilization to post-hatching. Stage one was observed only once; the description and time from fertilization until the midpoint of stage one is from Harrington (1963). A summary of the stages including the time from fertilization to the midpoint of each stage is given in Table 2.

Stage 1 (Figure 4a): The egg is fertilized and the perivitelline space is present. The blastodisc is present, resulting from the migration of cytoplasm to the animal pole of the egg. The yolk is transparent and contains a mass of oil droplets near the surface. This stage is rarely observed in rivulus because eggs are not released from the parental fish until after internal self-fertilization.

Stage 2: The first cleavage is meridional; the one cell dividing into two cells of equal size. Cleavage is meroblastic, thus the yolk mass does not divide. The surface of the egg has a speckled appearance which remains throughout development.

Stage 3: The second cleavage is meridional and at right angles to the first producing four clastomeres of approximately equal size.

Stage 4: The four blastomeres divide vertically to form eight blastomeres.

Stage 5: The blastomeres again divide vertically producing sixteen cells which, when viewed from the animal pole, are in four irregular rows of four cells each.

Stage 6: Cleavage is horizontal, producing thirty-two cells arranged in an upper and lower layer. The cells become progressively smaller as cleavage continues.

Stage 7: The dividing blastomeres have formed a low dome-shaped mass upon the yolk, the early blastula. Blastomeres are piled upon each other and it is hard to distinguish the separate cells.

Stage 8 (Figure 4b): The high blastula stage is recognizable by the distinct, bulging, bun-like dome of cells on top of the yolk mass.

Stage 9: During the late or flat blastula stage the blastomeres are still in a mass, but the margins of the blastoderm are less distinct and the dome-shaped mass is not as high as previously noted.

Stage 10: The expanding blastula begins gastrulation by epiboly and convergence. The margins of the blastoderm are ragged in appearance.

Stage 11 (Figure 4c): Gastrulation continues; the cell layer involved in epiboly, the epiblast, covers 1/3 of the yolk.

Stage 12: Gastrulation continuesl the epiblast covers 1/2 of the yolk. It is apparent that there is a massing of cells around the leading edge of migrating blastoderm, the germ ring. Also, there is a massing of cells in one area of the germ ring, the embryonic shield. The germ ring and the embryonic sheild are embryonic structures formed by the two movements of

Table 2. Summary of the Development Stages of *Rivulus marmoratus* at 25.0 ± 0.5 C, 14.0 ± 0.5 o/oo Salinity and 14 hours of Light per Day. The Times given are from Fertilization to the Midpoint of the Stages.

| Stage | Time (hours) | Description |
|---|---|---|
| 1 | 2.5 | 1 cell |
| 2 | 3.5 | 2 cells |
| 3 | 4 | 4 cells |
| 4 | 4.5 | 8 cells |
| 5 | 5.5 | 16 cells |
| 6 | 6.5 | 32 cells |
| 7 | 8 | Early blastula |
| 8 | 9.5 | High blastula |
| 9 | 10.5 | Late or flat blastula |
| 10 | 15 | Gastrulation begins, expanding blastula |
| 11 | 19 | Epiblast covers 1/3 yolk |
| 12 | 22 | Epiblast covers 1/2 yolk, germ ring and embryonic shield are forming |
| 13 | 24 | Epiblast covers 2/3 yolk |
| 14 | 25.5 | Large yolk plug, embryonic shield enlarging |
| 15 | 31 | Epiboly complete |
| 16 | 34.5 | Head and tail regions recognizable |
| 17 | 36 | Optic vesicles appear, somite formation begins |
| 18 | 43.5 | Optocoeles are prominent, auditory vesicles form |
| 19 | 53 | Optic cup and lens formation |
| 20 | 55.5 | Heart beats, no circulation |
| 21 | 58 | Body movement |
| 22 | 62 | Circulation |
| 23 | 71 | Increased vitelline circulation |
| 24 | 73 | Urinary bladder and otoliths first appear, pigmentation on brain, trunk, and yolk near embryo |

Table 2 (continued)

| Stage | Time (hours) | Description |
|---|---|---|
| 25 | 77 | Pectoral fins appear, otoliths are prominent |
| 26 | 90 | Liver first appears macroscopically, pigmentation on optic cup |
| 27 | 105 | Increased pigmentation and body movement |
| 28 | 140 | Pigmentation of the optic cup obscures the lens, circulation in pectoral fin and liver, caudal fin developing |
| 29 | 180 | Gas bladder and anal fin formation |
| 30 | 211 | Rays in caudal fin form, dorsal fin develops, the jaw appears |
| 31 | 240 | Pectoral fin movement, circulation in the caudal fin, a delay in hatching can occur |
| 32 | 310 | Hatching, rays in the dorsal and anal fin form; hatching may be delayed for days |

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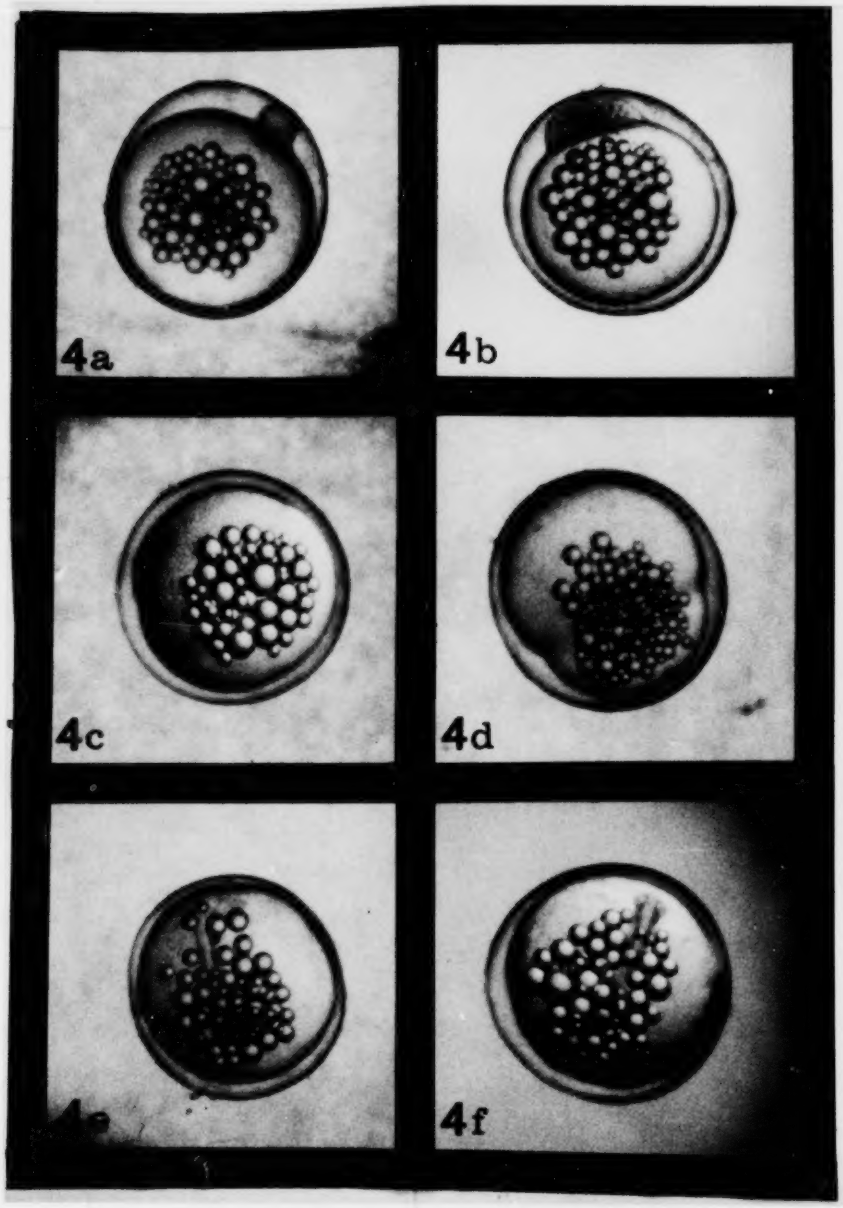

Figure 4a-f. Photographic representations of some early stages in the embryonic development of Rivulus marmoratus. Stages corresponding to figures 4a-f are: 4a-1, 4b-8, 4c-11, 4d-13, 4e-16, 4f-19.

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gastrulation, epiboly, and convergence, respectively. The mass of large oil droplets is present at the yolk surface near the germ ring.

Stage 13 (Figure 4d): Gastrulation continues; the embryonic shield enlarges; and the epiblast covers 2/3 of the yolk.

Stage 14: As gastrulation continues, the yolk material not yet covered by migrating epiblast cells bulges out forming a large yolk plug. Continued convergence of cells of the germ ring increases the embryonic shield longitudinally.

Stage 15: Gastrulation is complete; the epiblast encircles the entire yolk. The embryo, lying on the yolk surface, appears to be a furrow running through the mass of oil droplets located directly under the embryo. In oblique view, the embryo appears to be a translucent ridge curving around the surface of the egg.

Stage 16 (Figure 4e): The embryo continues to lengthen and amass tissue laterally and the head and tail regions of the embryo are recognizable. In this stage the embryo is seen as a prominent ridge on the surface of the egg.

Stage 17: The three regions of the brain, the forebrain, the midbrain, and the hindbrain, become visible and the optic vesicles appear as bulges off the forebrain. Late in this stage the optocoeles can be distinguished as cavities within the optic vesicles. The first few pairs of somites form, but are difficult to see due to the oil droplets massed below the embryo. The tip of the tail is bulbous, and there is a mass of tiny droplets directly posterior to the tail. The pericardial cavity forms extending on the yolk surface anterior to the head.

Stage 18: The embryo is increasing in size, and somite formation continues. The optocoeles are prominent within the optic vesicles and the auditory vesicles form lateral to the hindbrain. The pericardial cavity has increased in size and now extends anterior to and lateral to the head. The oil droplets are still beneath the embryo, but the head region now extends beyond the mass of oil droplets.

Stage 19 (Figure 4f): The optic region is developing with the formation of the optic cup and lens. The optic cup forms from the optic vesicle by invagination of the lateral surface of the vesicle; thus the optic cup appears first bean-shaped and then cup-shaped. The lens forms within the cup-shaped vesicle. The bulbous tip of the tail is slightly elevated off the yolk. The auditory vesicles are prominent, and the olfactory vesicles are visible. The expansion of the pericardial cavity, now extending from the tip of the head to the first somite, elevates that head. However, the tip of the forebrain is still flush with the yolk. There is increased brain development: the optic lobes appear as lateral enlargements of the midbrain and a furrow forms down the entire length of the brain.

Stage 20: The heart, visible within the pericardial cavity, extends from the yolk surface anterior to the embryo to the anteroventral surface of the embryo. This tiny tubular heart beats, but as yet no blood circulates.

Stage 21: The anterior somite area has sporadic contractions. Approximately 1/2 of the tail area is free of connection with the yolk, but the tail still lies close to the yolk. There is still a mass of tiny oil droplets posterior to the tail. Prior to circulation, anterior and posterior to the embryo, there is a massing of blood cells.

Stage 22: Circulation begins through the dorsal blood vessel and is observable anterior to the heart and posterior to the junction of the tail and the yolk. The vitelline vessels develop slowly; complete posterior-to-anterior vitelline circulation is established approximately two hours after the onset of circulation. As the vitelline circulation encompasses the entire yolk, the oil droplets, previously clumped together, spread outwards until they are envenly dispersed around the yolk. The optic lobes are enlarging, ventricles of the brain are observable, and the brain is taking on a coiled, convoluted appearance. There is a fine network of pigmentation covering the surface of the brain.

Stage 23 (Figure 5a): Circulation is well established now. The network of vitelline vessels is increasing; the vessels emerging from the embryo anterior to the somites are especially prominent. The brain continues to enlarge, and the optic lobes become massive and protrude anteriorly, overhanging the posterior portion of the optic cup. Pigmentation covering the embryo increases. The tail occasionally swings sideways.

Stage 24: Organ development increases now that circulation is established. A tiny urinary bladder forms dorsal to the hindgut and ventral to the dorsal blood vessel. The otoliths first appear as tiny specks in the auditory vesicles. Pigmentation on the brain and trunk region increases as well as on the yolk lateral to the embryo.

Stage 25: The otoliths are dense aggregations now, and the pectoral fins appear as tiny triangles protruding from the yolk surface immediately anterior to the first somite.

Stage 26 (Figure 5b): The pectoral fins lengthen until they project halfway up to the dorsal surface of the trunk. There is a general increase in pigmentation on the head, trunk, and tail of the embryo and on the yolk surface. Pigmentation shades the optic cup a light grey, but the cup is still transparent. The liver bud forms and can be seen on the left side of the embryo immediately posterior to the pectoral fin. Liver bud formation was not followed histologically; these are macroscopic observations on whole embryos.

Stage 27: The liver increases in size and becomes globular in appearance. Somatic pigmentation continues to increase, especially down the dorsal surface of the tail. The tail occasionally swings vigorously enough to touch the head.

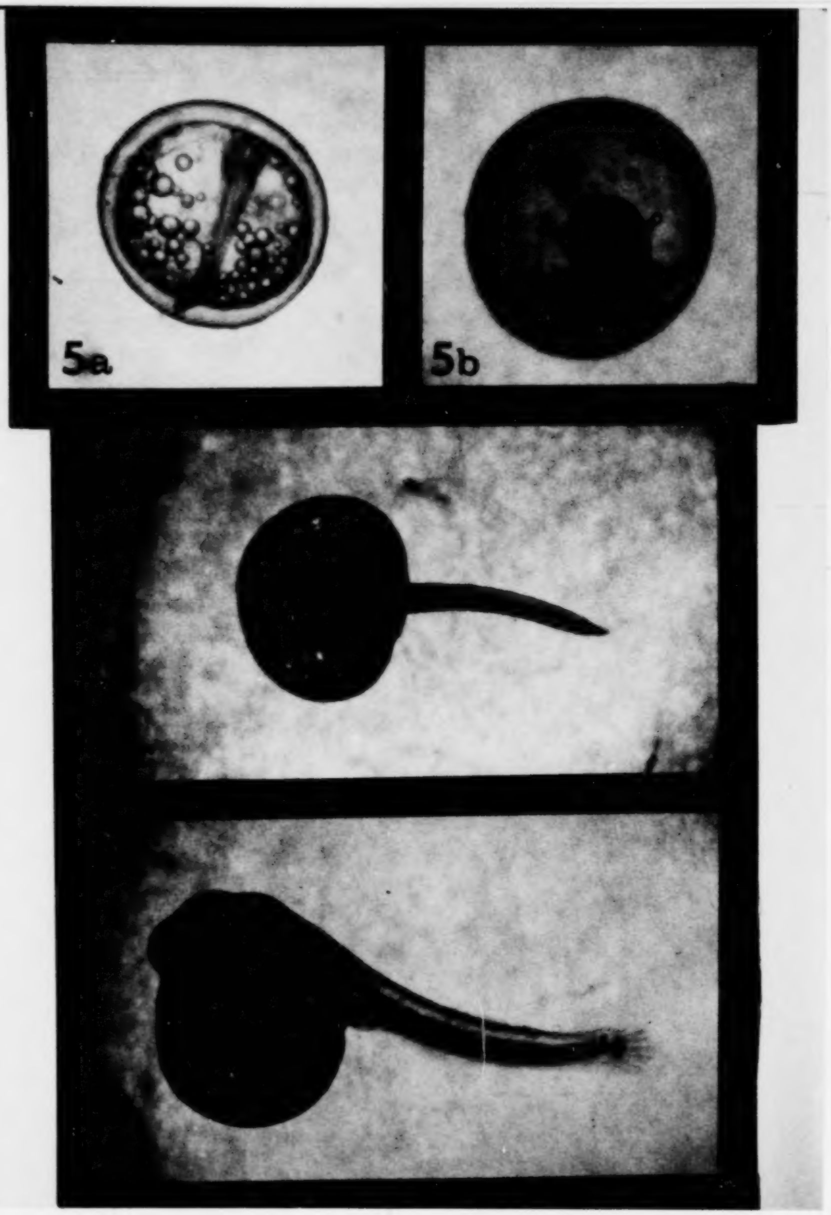

Figure 5a-d. Photographic representations of some late stages in the embryonic development of Rivulus marmoratus. Stages corresponding to figures 5a-d are: 5a-23, 5b-26, 5c-28, 5d-31. Chorions were removed from embryos 5c and 5d.

Stage 28 (Figure 5c): Pigmentation of the optic cup is so dark that it obscures the lends, and pigmentation of the peritoneum is obvious. The body cavity and liver are enlarging. The pectoral fins enlarge and circulation is observable in them. The caudal fin is forming, appearing as a transparent membrane around the posterior end of the caudal peduncle.

Stage 29: The body cavity is large, lifting the embryo off the yolk surface, but the grey twisting gut does not fill the body cavity. The gas bladder forms dorsal to the gut, but because of dark pigmentation it is hard to distinguish. The pectoral fins project as high as the dorsal surface of the trunk, and the anal fin begins developing. In the tail, circulation is observable between the caudal artery and vein.

Stage 30: The rays form in the caudal fin and the dorsal fin begins developing. Jaw formation is observable and the developing jaws are elevated off the yolk. Pigmentation is heavy, guanine is located along the vertebrae, on the bases of the pectoral fins, and on the surfaces of the optic cups.

Stage 31 (Figure 5d): The pectoral fins flutter slightly, but have no coordinated movement. Pigmentation is heavy on the dorsal surfaces of the head, trunk, and tail, on the yolk sac, and on the opercula. Circulation is established in the caudal fin. The anal fin is more pronounced than the dorsal fin, but no rays are present in either fin. A delay in further development sometimes occurs at this stage; the embryo can enter a period showing little or no movement and an irregular heart beat.

Stage 32: The mouth opens and closes with accompanying opercular movements, and the pectoral fins are motile. Rays develop in the dorsal and anal fins, and pigmentation appears along the rays of the caudal and pectoral fins. Hatching occurs at this stage.

The young fish actively swims from the moment of hatching. There is still a small amount of yolk observable on the ventral surface of the body. Although most eggs of rivulus hatch within 16 days after fertilization, some eggs may take up to 14 additional days after reaching stage 31 to hatch. Eggs of other oviparous cyprinodonts also have varying times of hatching. In different species the eggs may undergo either a period of latency or delay (Harrington, 1959) or a diapause (Wourms, 1972a, b, c). In diapause the embryo appears to be in a state of near suspended animation with little or no growth, development, movement, or yolk utilization. This may be an extended stage, the usual duration being two to three months for the annual cyprinodontid fish *Austrofundulus myersi*--Wourms (1972a). On the other hand, in a period of latency the embryo continues growth and utilization of the yolk. This delay is usually of short duration because the limited amount of yolk, among other things, would curtail an extensive delay. Koenig and Livingston (1976) showed that embryos of the cyprinodontid fish *Adinia xenica* could successfully delay hatching for ten days, the delay disrupted by a change in environmental conditions. Because rivulus exhibits a relatively short interruption of final development and continues to utilize the yolk during this period, it appears that it exhibits a delay or latency rather than a diapause. However, rivulus shows only a limited amount of activity during this latency period. Often during this period, the pulsations of the heart are so infrequent that no signs of circulation are observable; for example, blood cannot be seen moving through the vitelline vessels, usually sites of easily observed circulation. Often the heart will resume regularity after stimulus from the probe used in handling the eggs. However, hatching will not occur immediately after this increase in activity. The control of hatching or the cause of delayed hatching in rivulus is not known.

Some of the most interesting characteristics of rivulus cause difficulties when this fish and its eggs are used in research. Due to the fact that eggs are emitted in different stages and often not until after 10 hours of development (Harrington, 1963), it is difficult to observe the entire sequence of development, especially repeatedly, among a series of eggs. In addition, any study using rivulus as the test animal could run into difficulties if it were necessary to study the effects of some factor upon a certain age group. A large number of parental fish are necessary to obtain a large number of eggs or juveniles in the same stage of development.

Comparing the development of *R. marmoratus* to that of the commonly studied cyprinodont *Fundulus heteroclitus*--(Solberg, 1938; Armstrong and Child, 1965) shows that the overall sequence of development stages is the same, but that rivulus has a slower rate of development through some stages. In particular, brain and circulatory development in rivulus takes longer than it does in *F. heteroclitus*. Another developmental difference between these species is that the embryos of rivulus tend to acquire heavy pigmentation in the later stages of development. The pigmentation is noticeably darker than that of *F. heteroclitus*. Because of this heavy pigmentation it is sometimes difficult to distinguish development of internal organs such as the liver and swim bladder.

## COMPARISON OF EGG INCUBATION TECHNIQUES

Because many estuarine cyprinodontid fish deposit their eggs so that development takes place in moist air (Harrington, 1959; Hastings and Yerger, 1971; Able and Castagna, 1975; Taylor *et al.*, 1977; Johnson, 1980) a comparison of air incubation and water incubation was made. In a previous study (Shealy, 1979) air incubation was found superior to water incubation for embryos of *Fundulus heteroclitus*. Development was more rapid and more uniform in air incubated *Fundulus* eggs.

Twelve rivulus were held singly in reproduction chambers (25 ± 0.5 C, 14 hr photoperiod). Eggs were collected daily and a random number table was used to select the appropriate culture technique (odd = air, even = water). Water incubated eggs were held singly in 5 cm culture dishes filled to 1 cm with synthetic seawater (14 o/oo). Air incubated eggs were held singly in 5 cm culture dishes on three layers of Whatman$^R$ no. 2 filter paper which had been moistened with synthetic seawater (14 o/oo). When embryos reached the terminal stage of embryonic development (Stage 32) eggs were inundated for 1 hr with freshly collected seawater. If hatching did not occur within

1 hr, incubation conditions were resumed and the procedure was repeated the following day and so on until hatching occurred. The approximate age of each embryo was estimated by observing its stage of development upon or shortly after oviposition. All embryos were observed and staged daily under low light on a dissecting microscope.

Comparison of the terminal stages of embryonic development (Table 3) shows that development in water was more rapid than development in air although uniformity was approximately the same for both. The method chosen for egg development was therefore water incubation as described in the section "Laboratory Culture of *Rivulus marmoratus*".

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Table 3. Comparison of Rates of Embryonic Development of *Rivulus marmoratus* in Air Incubation vs. Water Incubation

| | Time(hrs)[a] | | | |
|---|---|---|---|---|
| Stage | Air | Water | t[c] | |
| 26 | 115.4 (4.66,14)[b] | 110.5 (4.37,11) | 0.75 | NS[d] |
| 27 | 141.6 (5.17,15) | 135.5 (4.61,8 ) | 0.77 | NS |
| 28 | 167.3 (6.43,8 ) | 149.8 (4.78,12) | 2.22 | $p < .05$ |
| 29 | 186.7 (4.95,22) | 176.0 (3.97,18) | 1.88 | NS |
| 30 | 236.3 (4.98,27) | 210.5 (5.22,12) | 3.12 | $p < .01$ |
| 31 | 277.6 (7.89,11) | 241.6 (5.59,16) | 3.84 | $p < .01$ |
| 32 | 291.2 (7.67,13) | 264.8 (9.15,12) | 2.22 | $p < .05$ |
| Hatch | 368.6 (6.98,13) | 355.4 (7.80,12) | 1.24 | NS |

[a] Average time from fertilization to midpoints of stages 26 through 31, and to the beginning of stage 32.

[b] Numbers in parentheses are standard errors and numbers of observations, respectively.

[c] Student's t

[d] NS = not significant

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# SECTION 5

# BEHAVIORAL BIOASSAY USING *RIVULUS MARMORATUS*

## INTRODUCTION

A recent review article by Cairns and van der Schalie (1980) summarized and discussed the concept of early warning systems in biological monitoring. These systems are charged with the responsibility of detecting environmental contamination at a very early stage so that further perturbation can be prevented and clean-up operations begun. Central to a biological early warning system is an organism possessing an observable physiological or behavioral trait which is rapid, quantifiable, and, most important, sensitive to harmful environmental variation. Of practical importance, the organism must be easy and inexpensive to culture if it is going to achieve widespread use. Numerous species are presently being employed in this capacity (see Table 1 in Cairns and van der Schalie, 1980). Yet, there is a constant search for more suitable species to replace or supplement these because, in most cases, one or more of the above conditions is not met satisfactorily. The objectives here are to describe a behavioral response by the cyprinodont fish *Rivulus marmoratus* to aquatic hydrogen sulfide contamination, and to evaluate its potential for use in a biological early warning system.

Recently *R. marmoratus* has been implicated as having terrestrial habits (Kristensen, 1970; Brockman, 1975; Courtney, 1975; Abel, 1981). Abel (1981) proposed a possible ecological basis for the terrestrial behavior which involves toxic levels of $H_2S$. In the laboratory, upon exposure to $H_2S$, *R. marmoratus* leaps from the water and either sticks to or lies on emergent objects, apparently respiring cutaneously for up to several hours before returning to the water. The unique ability of *R. marmoratus* to detect and avoid contaminated water in this way, plus the advantages of using genetically indentical individuals, serve as the bases for this bioassay.

Sulfurous effluents from industrial sources such as pulp mills, chemical and gas plants, and oil refineries burden many aquatic environments (Ellis, 1937; Van Horn *et al*., 1949; Dorris *et al*., 1960). In addition, $H_2S$ is produced from decomposition of sewage (Colby and Smith, 1967; Ziebell *et al*., 1970). Since $H_2S$ is an extremely potent metabolic poison of widespread occurrance, and because it has been demonstrated that *Rivulus marmoratus* detects and avoids toxic levels, $H_2S$ was selected as a reference toxicant for this study.

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## MATERIALS AND METHODS

Fish used in this study were maintained throughout in synthetic seawater (Instant Ocean$^R$ and distilled water) adjusted to 14 ± 1 ppt, with a 14 hr photoperiod, and at a constant temperature of 25 ± 1 C. Additional information on culture methods is given under the section "Laboratory Culture of *Rivulus marmoratus*" of this report.

Assays of $H_2S$ were conducted in covered, 400-ml Pyrex$^R$ beakers modified for flow-through capacity. An oyster shell attached to the side of each beaker slightly above the level of the overflow tube served as a platform onto which the fish could jump and lie. The experimental design contained numerous precautions to eliminate bias. Five beakers, each fed by a different head tank, were used in each run. To three randomly (by random number table) selected tanks different solutions of $H_2S$ were added. Two other randomly chosen tanks served as controls. Water pH was 7.9 ± 0.05 in all tanks. Except for one control which contained air-equilibrated water dissolved oxygen concentration was 2.0 ± 0.05 ppm in all tanks. Assays were run at low oxygen tension to duplicate conditions: (1) possibly associated with conversion to aerial respiration in the natural habitat of *Rivulus marmoratus* and, (2) frequently associated with the presence of $H_2S$ in other areas.

Five fish from 0.3 - 0.5 g, starved for three days, were randomly selected and individually added to a separate beaker containing 25 mℓ of normoxic water. The experimental solutions were then introduced at a rate of 2.0 ± 0.05 ℓ hr$^{-1}$, which was maintained throughout each run. The fish were allowed to acclimate for 1 hr, followed by 1 hr of observation. Each fish was used only once. Since preliminary experiments suggested that the ability of a fish to flop onto the oyster shell required learning, a positive response to the test solution was chosen to be not only (1) leaping from the water onto the shell, but also (2) demonstration of an attempt to leave the water as illustrated by a least one jump completely from the water. In addition to this quantal response, total time emergent and number of jumps were recorded. Eleven runs utilizing fifty-three fish were performed. A single observer scored all runs without knowledge of the relative or absolute $H_2S$ concentrations of the solutions.

Hydrogen sulfide and oxygen tensions were measured in water samples taken from the beakers at the beginning of acclimation, at least once during an experiment, and at the conclusion. Additionally, blanks were run and measured. Close monitoring is necessary since $H_2S$ oxidizes rapidly. However, since $H_2S$ concentrations varied $\leq$ 15% and $O_2$ did not change significantly over the time span, values of variables measured at the beginning of acclimation were used in all calculations. Solutions of $H_2S$ were prepared by adding calculated amounts of sodium sulfide to deoxygenated water. Hydrogen sulfide was assayed by the spectrophotometric method (American Public Health Association, 1976). Seawater was deoxygenated by bubbling nitrogen through it while monitoring oxygen tension with a Yellow Springs Instrument Corporation Model 51-A $O_2$ meter, calibrated prior to each use by the azide modification of the Winkler method (American Public

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Health Association, 1976).

Probit analysis (Finney, 1971) was used to analyze the quantal response of jumping. Data were grouped into intervals of 100 ppb $H_2S$ and the midpoints used in the calculations. Spearman's Coefficient of Rank Correlation (Steel and Torrie, 1980) was used to examine the relationship between actual $H_2S$ concentration and the amount of time fish responding positively remained emergent, that is, on the oyster shell.

## RESULTS

The concentration of $H_2S$ that elicited a positive response in 50% of the fish (Median effective concentration = EC50) was 123.59 ppb. The 95% confidence limits were 63.68 to 181.97 ppb. Figure 6 depicts the results of the assay. Of the twenty fish responding positively, sixteen actually jumped on the oyster shell. Analysis of the amount of time spent emergent (from 0 to 60 min) for the twenty fish by Spearman's Coefficient of Rank Correlation showed a significant positive correlation ($r = 0.52$, $p < 0.05$) between $H_2S$ concentration and time emergent. Data are presented in Table 4. There was no discernible relationship between $H_2S$ concentration and number of jumps. Of additional significance was an alternate strategy of avoidance exhibited by fish in the lower concentrations of $H_2S$. These fish aligned themselves at the air-water interface, apparently using the oxygen-microlayer, for as long as the duration of the run; controls did not exhibit such behavior. This behavior was not quantified. Although data on time to first leap were not collected since close observations were not made during acclimation, three fish were observed emergent at the beginning of the observation period. The solutions in which this occurred were 233, 252, and 579 ppb $H_2S$.

## DISCUSSION

This experiment demonstrates the ability of Rivulus marmoratus to detect an avoid water contaminated with $H_2S$. Although the EC50 determined by this investigation (123.59 ppb) was higher than those reported in the literature for other sub-lethal responses for example, Lepomis macrochirus exhibited decreased swimming endurance at 1.5 ppb $H_2S$ (Oseid and Smith, 1972) , it is sensitive enough to warrant more comprehensive testing of the leaping behavior as an indicator of $H_2S$ pollution. Quantification of other reactions, such as alignment at the surface or activity patterns, could result in a much more sensitive response. Also, since R. marmoratus is preadapted to still conditions (see below), static assays might lower the EC50.

Analysis of the amount of time emergent for those fish responding positively to the treatments demonstrated that at higher $H_2S$ concentrations, fish generally remained emergent longer. The high variability at the higher concentrations and the low correlation coefficient may be due to toxic effects of the $H_2S$.

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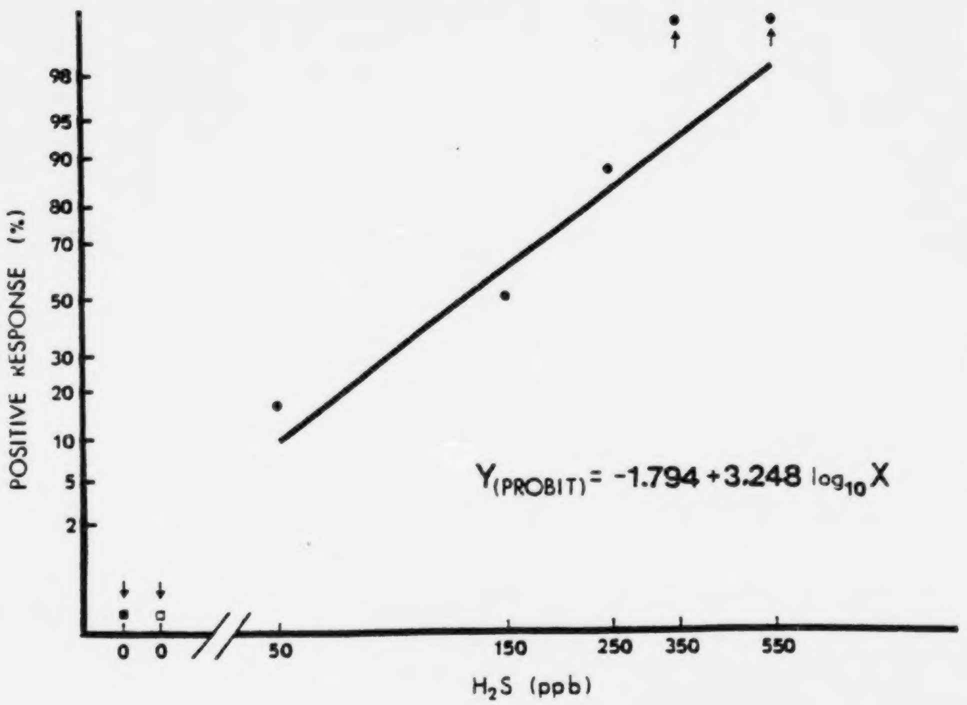

Figure 6. Log-probit plot of % positive response vs. $H_2S$ concentration. Solid dots represent experimental fish. Solid block is control run in air-equilibrated water. Open block is control run at 2.0 ppm $O_2$ concentration. Downward and upward arrows are directed at points representing 0 and 100% response, respectively. $H_2S$ concentrations are mid-points of 100 ppb intervals. Other conditions are described in the text.

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Table 4. Total Time Emergent in Minutes for Each of the Twenty Fish Responding Positively to the Treatments. Positive Correlation Between the Variables was Significant ($p < 0.05$)

| $H_2S$ Concentration (ppb) | Total Time Emergent (min) |
|---|---|
| 25 | 0* |
| 108 | 15 |
| 147 | 14 |
| 172 | 17 |
| 172 | 0* |
| 195 | 0* |
| 200 | 47 |
| 209 | 0* |
| 211 | 38 |
| 232 | 45 |
| 233 | 51 |
| 252 | 60 |
| 270 | 39 |
| 304 | 31 |
| 339 | 27 |
| 399 | 31 |
| 525 | 54 |
| 564 | 28 |
| 565 | 15 |
| 579 | 52 |

*Represents response judged positive based on jumping criterion (see text).

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Koenig and McLean (1980) discussed the manifold advantages of using the *Rivulus marmoratus* bioassay system relative to chronic marine bioassays. Here, the characteristics of the system will be represented as they pertain to biological early warning systems, using as guidelines the requirements for such systems as given by Cairns and van der Schalie (1980).

1. The behavior used in this bioassay is easily quantifiable through electronic devices, such as the one described by Cripe *et al*., (1975), which has been successfully tested for use with *Rivulus marmoratus*. Since avoidance removes the fish from physical contact with the toxicant, the fish is not noticeably stressed during the bioassay except at highly toxic levels of contaminant, such as possibly occurred in this experiment when some fish in the higher concentrations of $H_2S$ remained emergent less than fish in medium or low concentrations.

2. Although rapidity of response was not measured in this experiment, the response has been observed previously to be instantaneous to such toxicants as $H_2S$, ammonia, formalin, and rotenone, all in dilute solutions. That a large number of fish responded within 2 hours of the initial exposure in this study places the reactions in the upper 40% for the fish listed in Table 1 of Cairns and van der Schalie (1980). Although it is not known how wide a range of toxicants will elicit the jumping behavior, those which affect respiration or otherwise irritate the fish are obvious possibilities.

3. The response of *Rivulus marmoratus* to so-called non-harmful variation in water quality is not well-known. The species typically inhabits very shallow areas ( $\leq$ 15 cm deep) which are subject to wide variations in water level and salinity. In the laboratory, this species has been held in salinities of 0 (distilled water) to 50 ppt without noticeable avoidance behavior (it is therefore suitable for both freshwater and marine bioassays). As demonstrated here, environmental hypoxia does not induce jumping, since *R. marmoratus* possesses adaptations which have been shown by Lewis (1970) for other cyprinodontoids to facilitate respiratory use of the oxygen-rich surface microlayer.

4. An important characteristic of the system is genetic uniformity. Genetic variation should therefore not enter as a source of experimental variability. However, the data presented, particularly those in Table 4, indicate considerable variability; although behavioral responses of this nature are generally quite variable. As a future study it would be interesting to investigate directly the relationships between genetic uniformity and uniformity of experimental data.

5. Because *Rivulus marmoratus* inhabits still waters, it adapts well and quickly to living in culture dishes in the laboratory. Therefore, large numbers of fish can be maintained in small areas at low cost. In addition, fish older than 4 months produce a constant supply of eggs throughout the year.

Clearly, Rivulus marmoratus possesses qualities equaled by no other single fish species, freshwater or marine, for use in bioassays. Of course, there are also a number of possible disadvantages in the use of any single test animal and this depends on the particular experimental design. For example, homozygosity and isogenicity may be disadvantages to the experiment purporting to extrapolate to responses of heterozygous populations. Also, rivulus is quite aggressive and thus it is important that adults be held singly or be given refuges when held communally.

Although more comprehensive testing is required and appropriate interfacing techniques applied to complete the system, the use of R. marmoratus, including the novel application here, has promise in water quality management, and this species merits consideration for adoption as a standard behavioral bioassay organism.

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# SECTION 6

## CARCINOGENESIS BIOASSAY USING *RIVULUS MARMORATUS*

### INTRODUCTION

Much early work on neoplasms in fish grew out of an unexpected outbreak of liver cancer in cultured rainbow trout, *Salmo gairdneri*,from 1957 to 1960 (reviewed by Sinnhuber *et al*., 1977a). The cause of the disease was traced to aflatoxin contaminated diet. Subsequent studies demonstrated not only the great cancer producing potential of aflatoxins but also the high sensitivity of certain strains of rainbow trout to chemical carcinogens in general.

Many recent studies have focused on establishing the sensitivities of certain species of small aquarium fishes to known chemical carcinogens. Stanton (1965) was the first to use a small aquarium fish, the zebrafish *Brachydanio rerio*, in this type of experiment and he found that hepatic neoplasms could be induced in as short a time as eight weeks after diethylnitrosamine (DEN) was added to the aquarium water. Later, hepatocellular carcinomas were induced by various carcinogens in two more species of small aquarium fish, the guppy *Poecilia reticulata* and the medaka *Oryzias latipes* (Sato *et al*., 1973, Pliss and Khudoley, 1975, Ishikawa *et al*. 1975, Ishikawa and Takayama, 1979). In reviewing a number of studies involving the effects of chemical carcinogens on small aquarium fish Matsushima and Sugimura (1976) concluded that their use in studies involving chemical carcinogenesis has certain advantages over mammalian systems.

However, much more work must be done before small aquarium fish test systems are demonstrated superior to established rat and mouse systems and included in standard protocols.

The purpose of this study is to establish the sensitivity of *Rivulus marmoratus* to known chemical carcinogens so that its potential as a test animal for carcinogenic studies may be evaluated.

### MATERIALS AND METHODS

Routine culture methods and conditions were described under section 2, "Laboratory Culture of *Rivulus marmoratus*". The actual experimental setups differed somewhat from the routine methods to facilitate handling of the fish and to minimize chances of exposure of laboratory personnel to carcinogenic compounds. Several experiments were devised in which adults

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(> 6 mos.), larvae (< 1 mo.) or embryos were exposed via water to various concentrations of diethylnitrosamine (DEN). Other experiments involving water exposure of rivulus to 2-acetylaminoflourene (AAF) (with ethanol used as a carrier) and injection (i.p. with cod liver oil carrier) of benzo-a-pyrene (BaP) and AAF were inconclusive. Therefore, only the results of the DEN exposures will be reported here. DEN was purchased from Sigma Chemical Co. and BaP and AAF from Aldrich Chemical Co.

In all experiments fish were assigned to various exposure levels randomly (random number table). Temperature, photoperiod and salinity were 25 ± 1 C, 14 hr and 14 ± 1 ppt, respectively. Also, containers were cleaned and solutions were renewed weekly. Fish were fed brine shrimp nauplii or frozen adults three times a week. In all cases control fish were treated identically as exposed fish but without the inclusion of the carcinogen. All exposures were carried out in a light and temperature controlled environator outfitted with an exhaust fan. Contaminated air from the environator was periodically purged via the laboratory hood and all transfers and cleanings were done under the hood. As an added precaution rubber gloves and a carbon filter gas mask were worn during feeding or cleaning. Any concentrated waste was stored in a stoppered glass jar under the hood and disposed of through the hazardous waste disposal procedure at the Medical University of South Carolina (MUSC), Charleston, SC. Because of our intent to compare the response of rivulus to other fish exposed similarly, concentrations were chosen on the basis of similar published experiments for DEN (Ishikawa and Takayama, 1979). Exposures continued until fish showed signs of toxic stress. In the case of the embryo exposures experimental data were lacking in the literature. Exposure levels were therefore designated in a log series up to the approximate lethal level. The three exposure experiments are described individually:
(1) Adult rivulus (30 fish per concentration) were exposed to DEN in the culture water in concentrations of 45, 30, 15, and 0 mg/ℓ for 5 weeks, then held in uncontaminated water for one year before being killed, examined grossly and prepared for histological examination. Fish were held in groups of 10 in 20.3 cm diam culture dishes which were kept covered in the environator. (2) Larval rivulus (30 fish per concentration) were exposed to DEN in the culture water in concentrations of 45, 30, 15, and 0 mg/ℓ for 12 weeks, then held in uncontaminated water for 1 year before being killed, examined, and prepared for histological examination. Each group of 30 larvae were held together in 20.3 cm diam culture dishes which were kept covered in the environator. (3) Embryos of rivulus (56 per concentration) were exposed to DEN in the culture water in concentrations of 1,000, 320, 100, 32, 10, and 0 mg/ℓ for 1 week. The resulting hatchlings were then held for approximately one year in uncontaminated water before being killed, examined, and prepared for histological examination. Prior to exposure embryos were staged and assigned to one of five groups:
(1) stages 1-9, (2) stages 10-14, (3) stages 15-17, (4) stages 18-23, (5) stages 24-31. See under section 3 "Embryonic Development". During exposures embryos were held in 20 ml screw top test tubes sealed with teflon liners. Not more than 5 embryos were held in each test tube. All exposures were carried out in the environator.

The major emphasis in this study was on induction of hepatocellular carcinoma. Therefore, fish were killed in 10% buffered formalin solution and the livers were dissected from the fish and processed according to standard histological procedures. Only fish that survived to the end of the experiment were included in the analysis. All livers and occasionally whole bodies were sectioned at several levels. Sections (5-6 µm) were then stained with hematoxylin and eosin (H+E). Slides were examined and tumors described by Dr. J. C. Harshbarger (Registry of Tumors in Lower Animals, Smithsonian Institution) and confirmed by Dr. A. B. Garvin (Medical University of South Carolina, Charleston, SC).

A single random section through all livers was used to determine tumor incidence. It was assumed that the source of error using this method (as opposed to serial sections) was small compared to experimental error.

## RESULTS

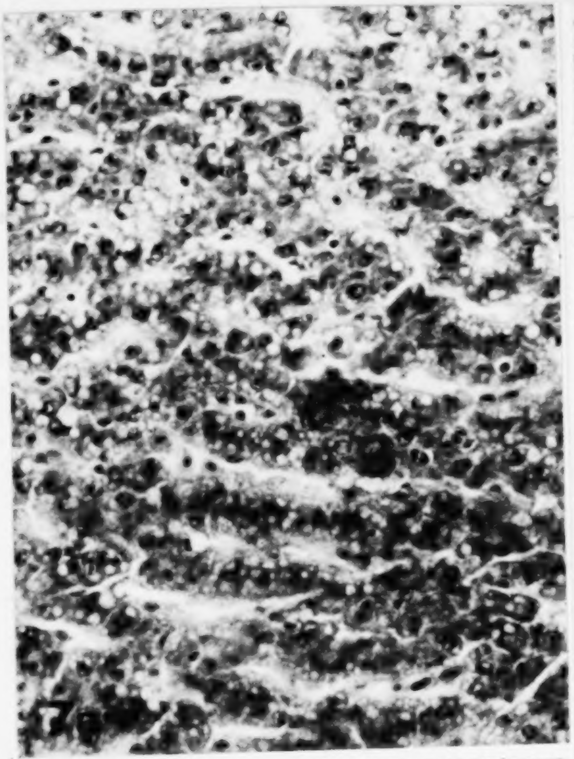
Histologically, the livers of rivulus resemble those of other bony fishes (Figs. 7a, 7b). Liver cords are two cells thick, rather than one as in mammals, and pancreatic tissue occurs within the liver along vessels rather than as a separate organ. No pathologic changes were observed in the livers of the control fish.

In DEN treated fish from the larval and adult exposure experiments, neoplastic changes originated from both hepatocytes and cholangiocytes. Hepatocellular neoplasms appeared mostly as basophilic nodules, but eosinophilic nodules were also present. Neoplastic nodules showed several patterns: solid (Figs. 7c, 7d), trabecular (Figs. 8a, 8b), and glandular patterns (Figs. 8c, 8d). Different patterns occasionally occurred together in the same liver. Boundries between tumor nodules and normal liver were distinct in some cases (Figs. 9a, 9b) but no thick fibrous capsules were seen. Other nodules lacked a distinct boundary with tongues of tumor extending into the normal tissue (Figs. 9c, 9d). In the adult exposures, patterns of hepatocellular carcinoma were of all three types of about equal proportions, however, solid and glandular patterns were much more common than the trabecular pattern in the larval exposures.

Tumor cells were enlarged with enlarged nuclei and distinct nucleoli. Size varied from only slightly to considerably larger than normal. Most tumor cells were fair to well-differentiated except in one case where many tumor cells were spindle-shaped (Figs. 10a, 10b). No tumor cells were so poorly differentiated that all resemblance to normal liver was absent.

Two types of ductule lesions were seen. One type, cholangioma (Figs. 10c, 10d) consisted of multiple, thick walled, various-sized, randomly oriented ducts or tubules formed by a single layer of tall columnar epithelium. In some ducts, epithelial papillae extended into the lumens. Fibrous stroma was minimal. The other type of ductule lesion had multiple, thin walled ducts or tubules formed by simple cuboidal or squamous epithelium (Figs. 11a, 11b). They tended to have larger ducts surrounded

Figures 7a-d. Photomicrographs of histological sections (6 µm, H & E) of the livers of *Rivulus marmoratus*. (a & b) Control fish showing normal liver cells with varying degrees of staining from dark (a) to light (b), x400. (c & d) Fish exposed to 45 ppm DEN for 12 weeks with solid pattern hepatocellular carcinoma, x200 and 400, respectively. Note enlarged cells and nuclei with prominent nucleoli.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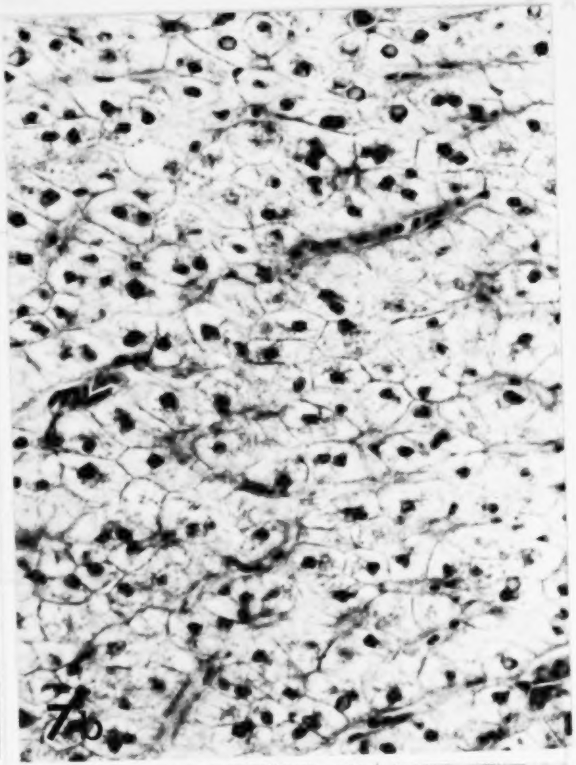
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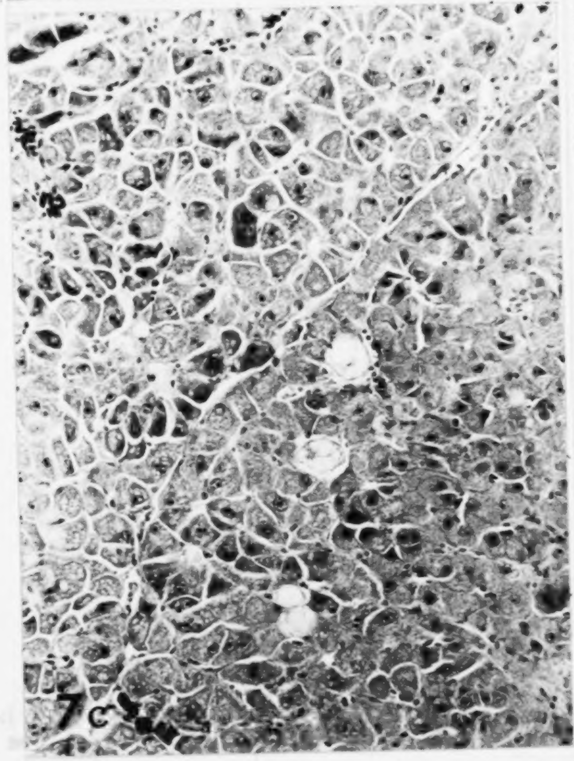
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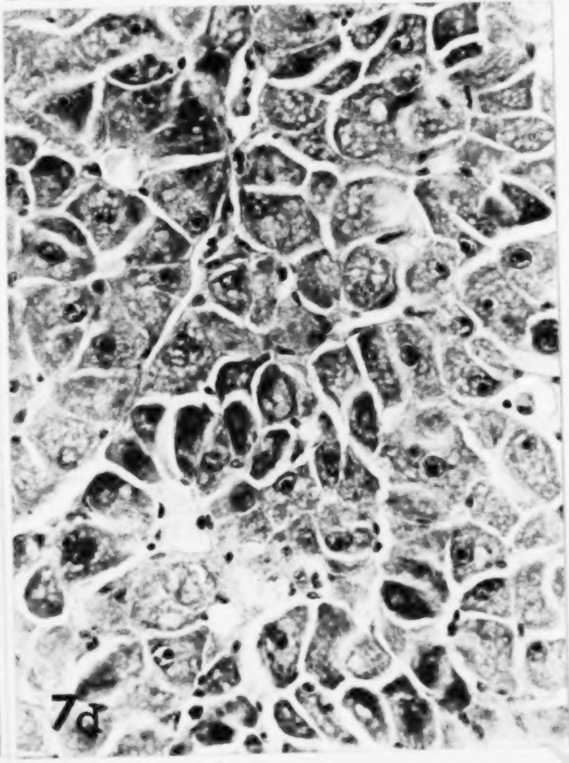
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Figures 8a-d. Photomicrographs of histological sections (6 μm, H & E) of the livers of Rivulus marmoratus; larvae were exposed for 12 weeks to 45, 30 or 15 ppm DEN then held in uncontaminated conditions for 1 year.
(a & b) Trabecular pattern hepatocellular carcinoma, x200 and 400, respectively.
(c & d) Glandular pattern hepatocellular carcinoma, x200 and 400, respectively.

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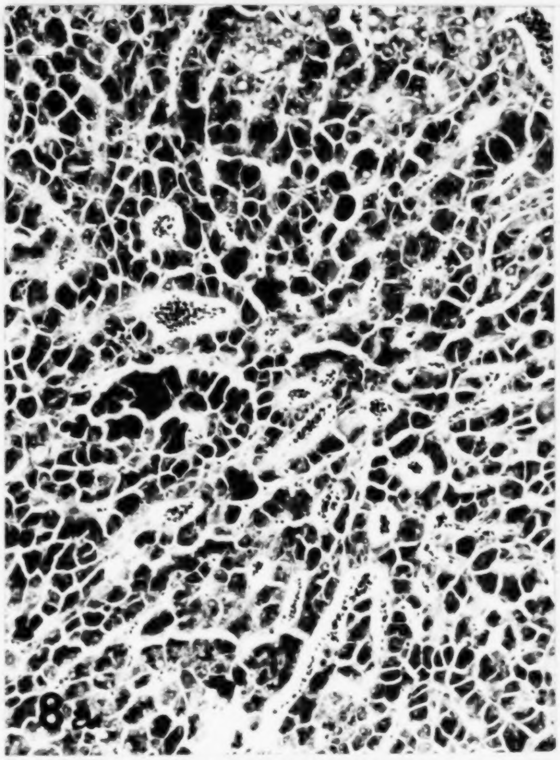

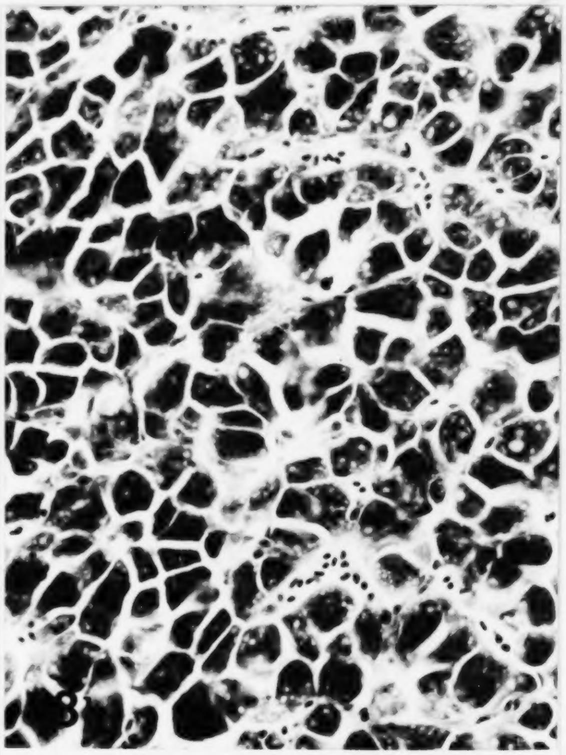

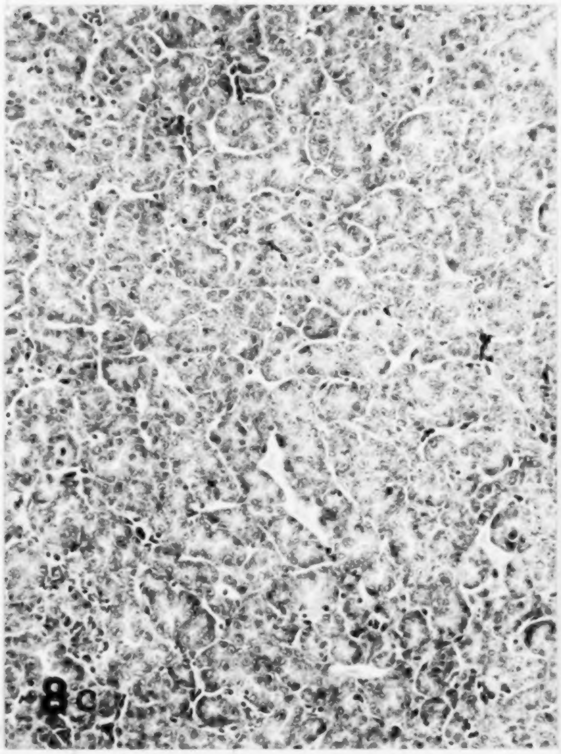

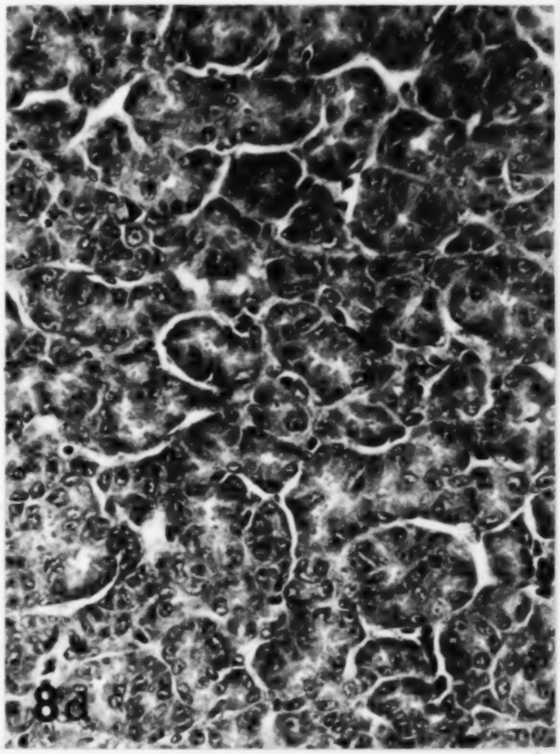

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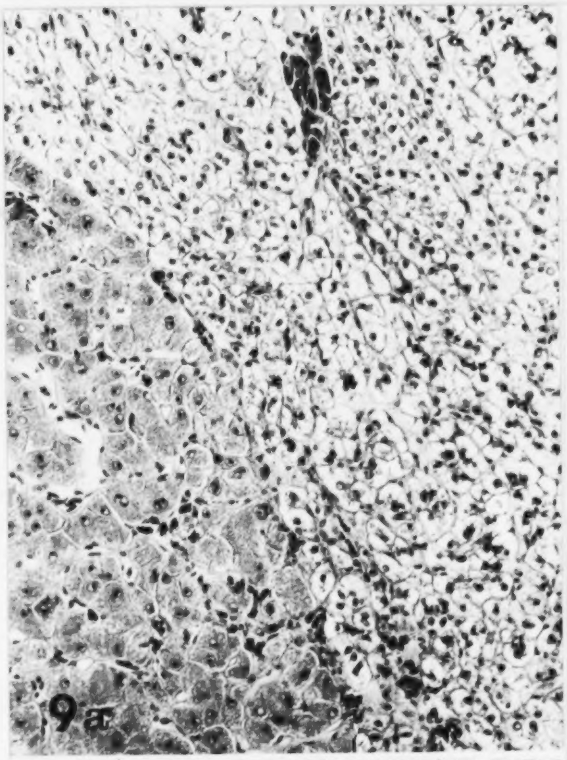
Figures 9a-d. Photomicrographs of histological sections (6 μm, H & E) of the livers of *Rivulus marmoratus*; larvae were exposed to 45, 30 or 15 ppm DEN then held for 1 year in uncontaminated water. Sections show distinct boundries between solid pattern tumor (a) and normal tissue(above)and trabecular pattern tumor (b) and normal tissue (above) x200. Indistinct boundries occur between glandular pattern tumor (c & d) and surrounding normal parenchyma, x400 and 200, respectively.

Solid x200

Trabec x200



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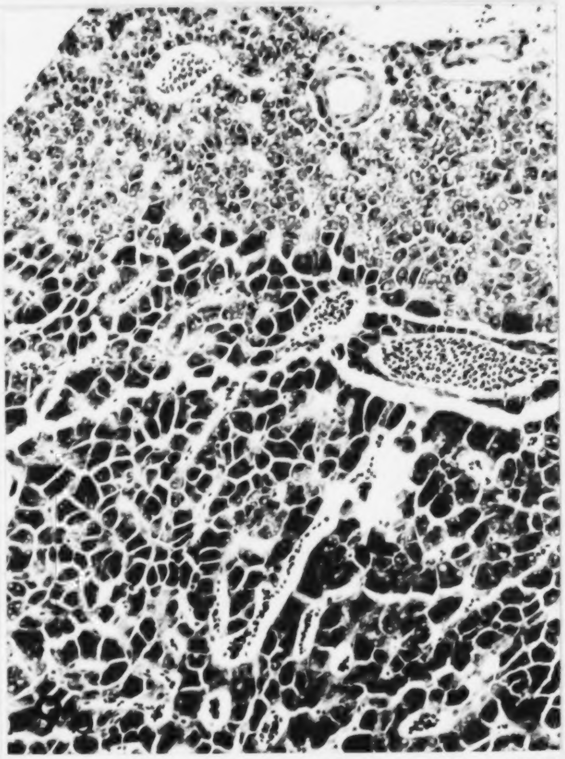

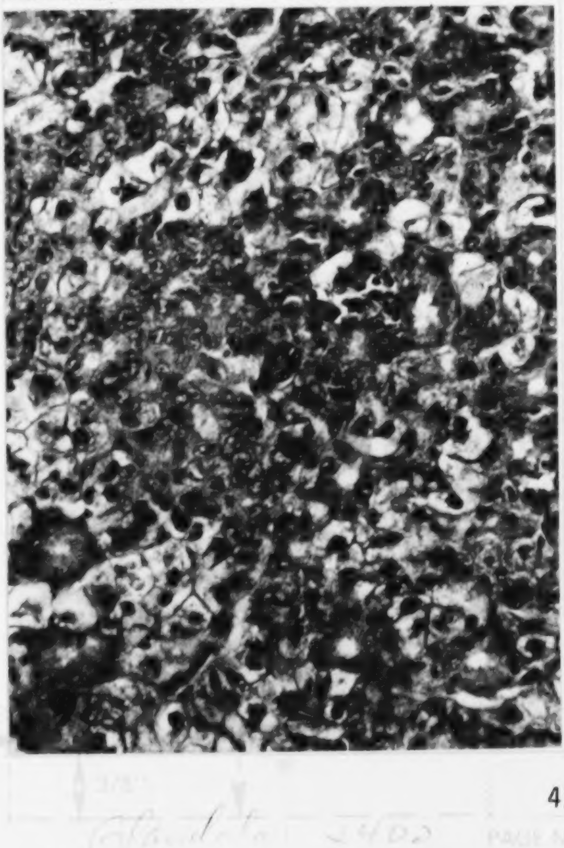

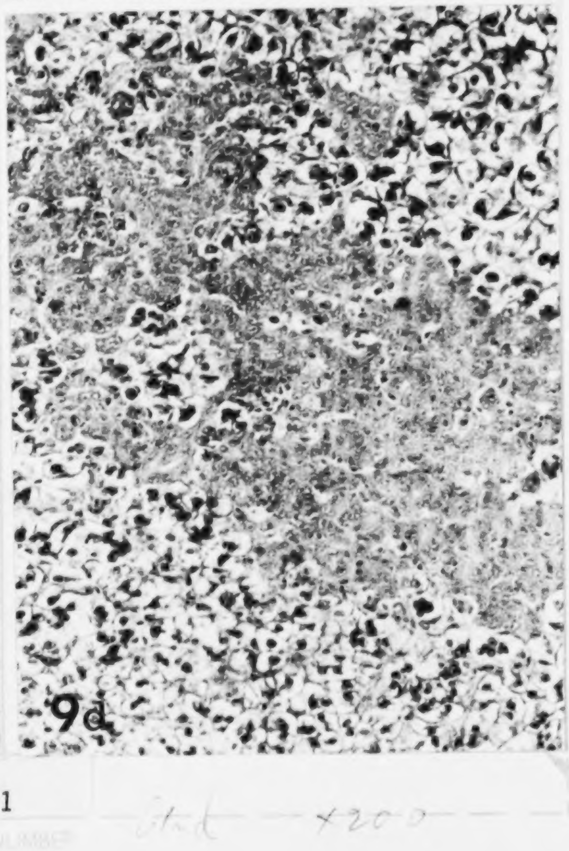

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Glandular x200

Std x200

Figures 10a-d. Photomicrographs of histological sections (6 μm, H & E) of the livers of *Rivulus marmoratus*; larvae were exposed to 45, 30, 15 ppm DEN for 12 weeks then held for 1 year in uncontaminated water. (a & b) tumor composed chiefly of spindle-shaped cells, x100 and 400 respectively. (c & d) cholangioma, x200 and 400, respectively.

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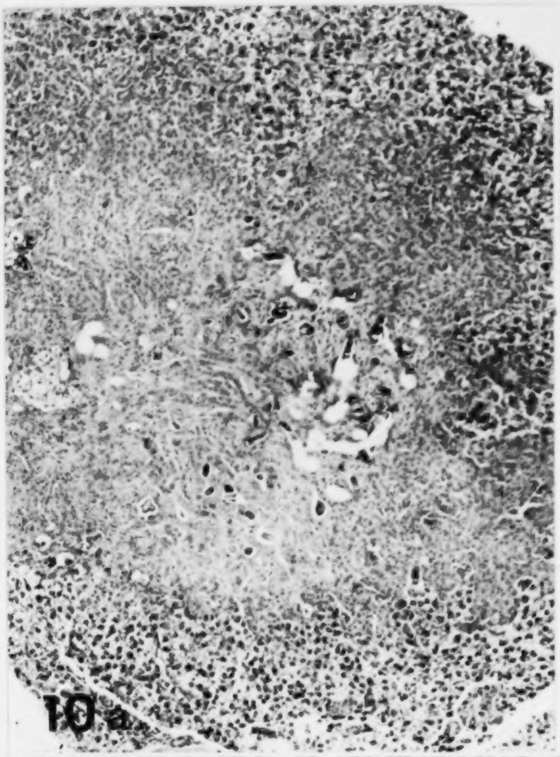
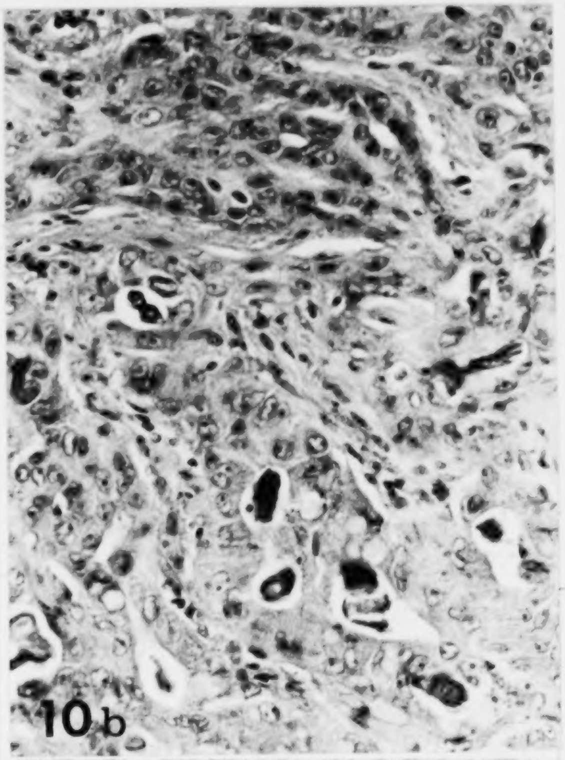
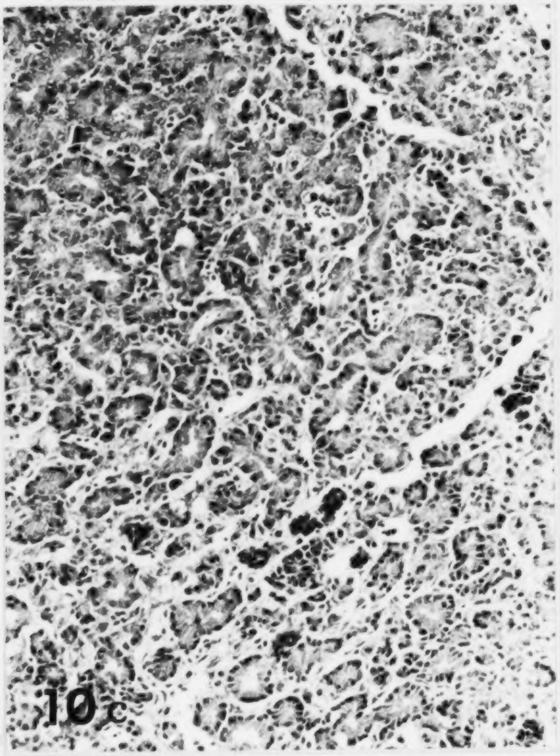
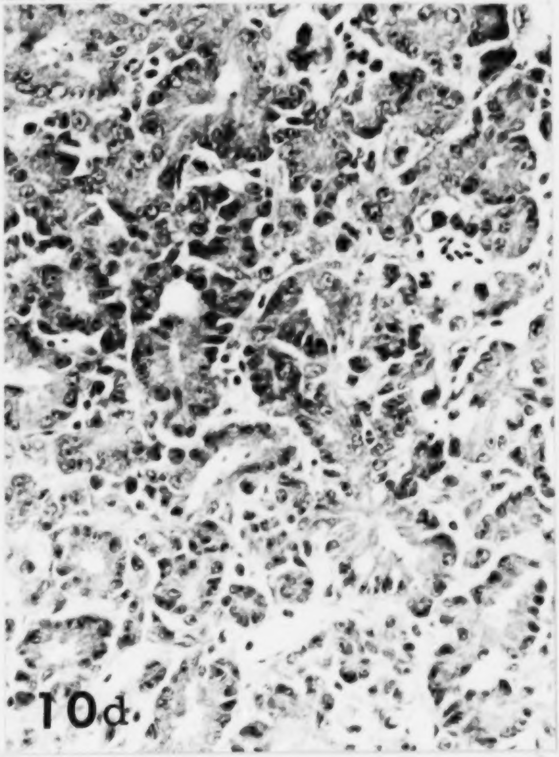
Spind ×400









Spind ×400

Figures 11a-c. Photomicrographs of histological sections (6 μm, H & E) of the livers of Rivulus marmoratus; larvae were exposed to 45, 30, or 15 ppm DEN for 12 weeks then held for 1 year in uncontaminated water. (a & b) Adenofibrosis, x 100 and 200, respectively. (c) Granuloma.

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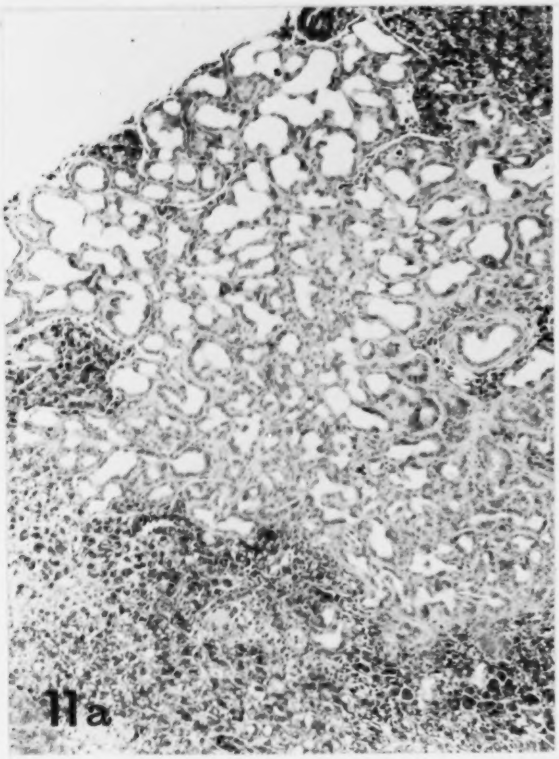

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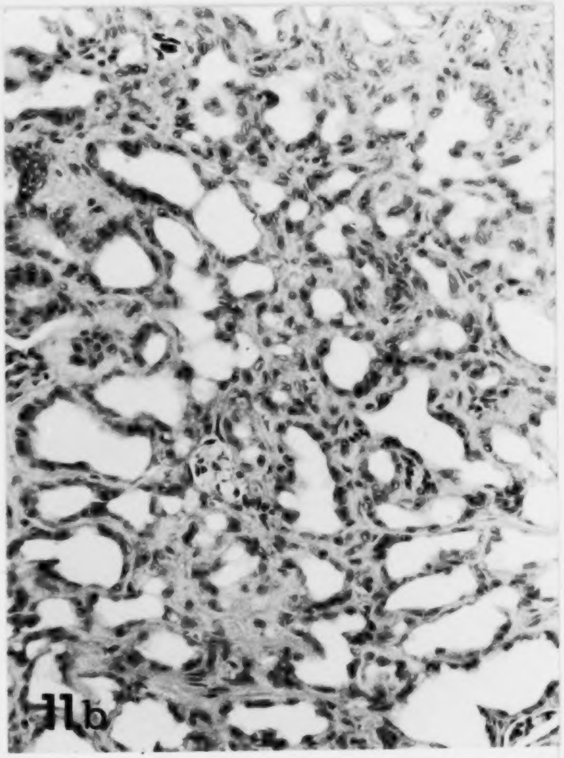

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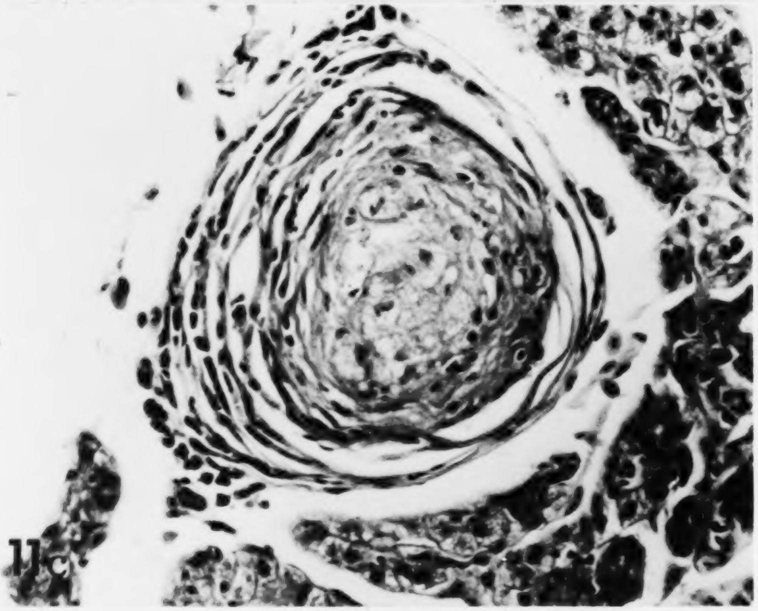

11c

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by smaller satellite ducts. The satellite ducts are considered metaplastic duct formations from the liver parenchyma rather than bile duct proliferations. Most ducts were ensheathed by fibrous tissue which was also sometimes abundant in the interductule spaces. These lesions were interpreted as adenofibrosis. Ducts within both types of ductule lesions, cholangioma and adenofibrosis, contained occasional concretions of unknown composition.

Chronic inflammatory lesions included granuloma (Fig. 11c), focal collections of epithelioid leukocytes with a fibrous sheath. In some livers, chronic inflammatory lesions were diffuse rather than focal as in granuloma. These often contained larger numbers of eosinophils. Macrophage centers composed of foamy macrophages containing a golden pigment were also fairly common.

There was no clear dose-response among the various pathologic changes; however, the incidence of hepatocellular carcinoma generally increased with exposure level for both larvae (Table 5) and adults (Table 6). Generally, in the two highest exposure levels of the larval experiment tumors tended to be large, occupying nearly all of the liver on the sections. Also, the numbers of tumors per liver section generally increased with dose level.

No pathologic changes were observed in the livers of fish exposed to DEN as embryos.

No neoplastic changes were found in organs other than the livers although a complete examination has not been done.

## DISCUSSION

This study clearly shows that DEN, a potent liver carcinogen in experimental mammals and fish, induces hepatocellular carcinoma, among other pathologic changes, in the liver of rivulus. Because of limited numbers of animals available for this study a staggered termination of the experiment was not done. A latency period of one year was chosen to avoid the ambiguity involved in the characterization of early neoplastic foci. Therefore, it is not known whether clear determination of hepatocellular carcinoma could be made with a shorter induction period.

The lack of hepatocellular carcinoma in rivulus treated with DEN as embryos is difficult to explain as fish embryos are considered more sensitive to hepatocarcinogens than are larvae or adults. For example, Wales et al. (1978) induced liver cancer in rainbow trout by exposing fertilized eggs to aflatoxin $B_1$ (0.5 ppm) solutions for 1 hour. The tumor incidence, determined 1 year after exposure, increased as the age of the embryo, during which the exposure was conducted, increased with the most dramatic rise occurring near the time of liver bud formation. Perhaps the lack of response of rivulus in this study relates to the possible impermeability of fish eggs to DEN, as suggested by Hendricks et al. (1980). However, in a later experiment liver tumors were induced in trout by increasing the embryo exposure level (J. D. Hendricks, personal communication).

Table 5. Pathologic Changes in Livers of Rivulus marmoratus Larvae Exposed to Diethylnitrosamine for 12 Weeks

| Nominal Water Concs. (mg/ ) | No. Fish (Start) | No. Fish (End)[a] | Fish with Neoplasms[b] | Incidence[c] | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Hepatocellular Carcinoma | Incipient Neoplasms | Cholangioma | Adenofibrosis | Granuloma |
| 0 | 30 | 23 | 0/23(0)[d] | 0/23(0) | 0/23(0) | 0/23(0) | 0/23(0) | 0/23(0) |
| 15 | 30 | 18 | 17/18(94.4) | 12/18(66.7) | 7/18(38.9) | 4/18(22.2) | 16/18(88.9) | 11/18(61.1) |
| 30 | 30 | 14 | 12/14(85.7) | 9/14(64.3) | 4/14(28.6) | 1/14(7.1) | 6/14(42.9) | 7/14(50.0) |
| 45 | 30 | 17 | 16/17(94.1) | 13/17(76.5) | 4/17(23.5) | 7/17(41.1) | 14/17(82.4) | 7/17(41.2) |

[a] Fish were killed one year after end of exposure period.

[b] No. of fish with neoplasms (hepatocellular carcinoma, cholangioma or incipient neoplasms)/total no. of fish.

[c] No. of fish with pathologic change/total no. fish.

[d] Nos. in parentheses are percents.

Table 6. Pathologic Changes in Livers of *Rivulus marmoratus* Adults Exposed to Diethylnitrosamine for 5 Weeks

| Nominal Water Concs. (mg/ ) | No. Fish (Start) | No. Fish[a] (End) | Fish with Neoplasms[b] | Incidence[c] | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Hepato-cellular Carcinoma | Incipient Neoplasms | Cholangioma | Adeno-fibrosis | Granuloma |
| 0 | 30 | 23 | 0/23(0)[d] | 0/23(0) | 0/23(0) | 0/23(0) | 0/23(0) | 0/23(0) |
| 15 | 30 | 20 | 0/20(0) | 0/20(0) | 0/20(0) | 0/20(0) | 1/20(5.0) | 0/20(0) |
| 30 | 30 | 21 | 7/21(33.3) | 6/21(28.6) | 2/21(9.5) | 1/21(4.8) | 13/21(61.9) | 5/21(23.8) |
| 45 | 30 | 16 | 5/16(31.3) | 5/16(31.3) | 0/16(0) | 0/16(0) | 8/16(50.0) | 2/16(12.5) |

[a] Fish were killed one year after end of exposure period.

[b] No. of fish with neoplasms (hepatocellular carcinoma, cholangioma, or incipient neoplasms)/total no. of fish.

[c] No. of fish with pathologic change/total no. of fish.

[d] Nos. in parentheses are percents.

The response of rivulus to DEN was not nearly as dramatic as that of the medaka. Our exposure levels (45, 30, 15 ppm) were the same as Ishikawa and Takayama's (1979) and the exposure durations and conditions were somewhat comparable; however, the medaka in their experiment showed tumors as early as 5-8 weeks after exposure to DEN ended and tumors were obvious on gross examination after 13 weeks. Medaka usually died of massive abdominal tumors after 20 weeks.

Although the DEN induced pathologic changes in livers of rivulus are similar to those of other experimental animals (Schmähl and Osswald, 1967), rivulus has several distinct advantages as a test animal for studies involving carcinogenesis. First, genetic uniformity of clones eliminates genetic variability as a source of experimental error thus giving better comparability of data between experiments and between laboratories (Sonntag, 1977). Because self-fertilization is the natural mode of reproduction, no special techniques are necessary to maintain isogenicity. Second, studies involving tissue transplants are possible. Third, the full life cycle is carried out in the simplest of culture systems, stackable glass culture dishes. No aeration is necessary so volatile chemical carcinogens, such as DEN, are not purged from the system. Fourth, eggs are emitted daily throughout the year under appropriate conditions of temperature, daylength and feeding. Thus, egg exposure experiments such as those described by Sinnhuber _et al_. (1977b) can be performed at all times of the year.

## SECTION 7

## TERATOGENICITY BIOASSAY AND CHRONIC FULL LIFE CYCLE EXPOSURE OF RIVULUS MARMORATUS TO SUBLETHAL LEVELS OF SELECTED TOXICANTS

### INTRODUCTION

The coastal and marine environments have become contaminated with a wide variety of organic and inorganic pollutants (Whittle et al., 1977). Many of these substances are not only toxic but are known carcinogens, mutagens, and teratogens; however, the potential effects (or the interacting effects) of the vast majority are unknown (Kraybill et al., 1977). One way in which to gain insight into the potential effects of these contaminants on marine organisms (and indirectly on the human population) is to establish a number of sensitive yet simple bioassays. The purpose of this study is to explore the feasibility of using the marine fish Rivulus marmoratus as a bioassay animal in the evaluation of the teratogenic and chronic sublethal effects of selected organic pollutants.

The advantages of using the self-fertilizing rivulus in this type of bioassay are: 1, homozygous, isogenic lines (Kallman and Harrington, 1964; and Harrington and Kallman, 1968) should allow a high degree of uniformity and reproducability in experimental results; 2, sensitivity to environmentally (temperatures) induced skeletal abnormalities (Harrington and Crossman, 1976) provides a convenient endpoint for the determination of teratogenic potential of various pollutants; 3, the extreme hardiness of rivulus under simplest of culture conditions (stackable glass culture dishes) allows full life cycle exposures to moderately high levels of various pollutants without excessive mortality, thus allowing observation of pathologic changes (Koenig and McLean, 1980). This combination of sensitivity and hardiness makes rivulus particularly attractive as a bioassay animal.

The selected chemicals used in this study are pentachlorophenol, 2, 3, 4, 6-tetrachlorophenol, 2, 3, 5-trichlorophenol, di-n-butyl phthalate and bromoform. Pentachlorophenol and 2, 3, 4, 6-tetrachlorophenol and their salts are widely used as biocides in the paint, wood and textile industries. They enter the marine environment directly as biocides in drilling and completion fluids for oil well drilling (see Rao, 1978). Also many chlorophenols are inadvertently produced in the chlorination of sewage effluents (Jolley, 1975). The toxicity, bioaccumulation potential and various other effects of pentachlorophenol and related compounds on aquatic organisms have been investigated (Tagatz et al., 1977; Pruitt et al., 1977; Glickman et al., 1977; Rao, 1978; Iwama and Greer, 1979; Manumante and

Kulkarni, 1979). Di-n-butyl phalate (DBP) is used abundantly as a plasticizer in the manufacture of polyvinyl chloride and other plastics. It has been found in marine (Giam *et al.*, 1978) and freshwater (Mayer *et al.*, 1972) environments and has been shown to be teratogenic in rats (Singh *et al.*, 1972). The toxicity, bioaccumulation potential and various other effects of DBP on aquatic organisms have been investigated (Sanders *et al.*, 1973; Pfuderer and Francis, 1975; Laughlin *et al.*, 1978; see Zitko, 1972 and Autian, 1973 for reviews). Marine bromoform contamination results primarily from the chlorination of seawater such as waters in cooling power plant condensers (Carpenter *et al.*, 1980). Bioaccumulation potential and toxicity to various aquatic organisms has been investigated (Gibson *et al.*, 1980; Trabalka *et al.*, 1980).

## MATERIALS AND METHODS

All culture methods and conditions were as described under Section 2 "Laboratory Culture of *Rivulus marmoratus*".

### Preliminary LC50 Tests

Sublethal water concentrations for the full life cycle chronic study were chosen as fractions (0.20, 0.10, 0.05) of static 96-hr LC50 tests (American Public Health Association, 1976) on newly hatched postlarvae of *Fundulus heteroclitus* and *Rivulus marmoratus*. The term postlarvae as used here refers to hatchlings less than two weeks old. Because of the difficulties initially in obtaining large numbers of postlarval rivulus in similar stages of development *F. heteroclitus*, a species in the same family, was substituted for the LC50 tests and chronic exposure levels were based on these. Later, smaller numbers of rivulus were tested to corroborate the LC50 data obtained initially on *F. heteroclitus*. Postlarvae were chosen because fish larvae are generally more sensitive to toxic effects than are either adults or eggs (except in the sense of Danil´chenko, 1977). Toxic concentrations based on toxicity to larval stages would therefore probably be sublethal for full life cycle exposures yet high enough to establish a quantifiable frequency of chronic effects. Postlarval *F. heteroclitus* were obtained from adults collected during the breeding season (March - September). After capture, the fish were brought to the laboratory and injected at the base of the dorsal fin with 50 uℓ (50 IU) of human chorionic gonadotropin in saline solution to stimulate gonad development. The fish were then separated by sex and held under a 14 hr photoperiod at about 25 C. Five to six days after injection the testes of several males were removed and minced in 10 mℓ of sea water (28 o/oo) in a 11.4 cm diam culture dish. Eggs were immediately stripped from females directly onto the sperm suspension. After about 10 min of gentle agitation the eggs were washed with fresh seawater and placed on moistened filter paper pads (5 layers of Whatman$^R$ no. 2) in 11.4 cm diam culture dishes. Dishes were covered to prevent dessication and kept in an environator at 25 C and a 14 hr photoperiod. After about 10-12 days of incubation embryos were hatched by flooding them with freshly collected water from Charleston harbor. Larvae were fed brine shrimp nauplii until they were used for the toxicity experiments.

Pentachlorophenol (99%) was purchased from Eastman-Kodak Chemical Company. 2, 3, 4, 6-tetrachlorophenol (technical grade), 2, 3, 5-trichlorophenol (97%) and dibutyl phthalate (99%) were purchased from Aldrich Chemical Company and bromoform (99%) was purchased from Fisher Scientific Company.

Static LC50 tests were run in 11.4 cm diam culture dishes for non-volatile chemicals or air-tight specimen jars of about the same volume for bromoform. Preliminary gas chromatographic analyses were performed to determine loss of bromoform from a 10 mg/ℓ solution over time. In tightly stoppered containers with little air space there was less than 10% loss over 24 hrs. The high concentrations of bromoform necessary for LC50 determinations were formed by sonification. All test chemical solutions were made fresh and changed every 24 hrs and postlarvae were fed brine shrimp nauplii daily to simulate conditions in the chronic study and to avoid cannabalism. A maximum of 10 postlarvae were randomly assigned to each container; however, there were as many as 5 containers per concentration of toxicant. Bromoform was most difficult to assess in terms of static LC50 because of its volatility and also because it narcotized the fish; that is, postlarvae judged as dead would regain equilibrium and activity soon after being placed in clean water.

Whenever possible LC50 tests were done on rivulus postlarvae from the same clone; however, it was later noted that differences in response between clones were insignificant relative to other variables involved with the tests. Probit analysis (Finney, 1971) was used when possible to evaluate dose-mortality curves otherwise graphic methods were used.

## Chronic Exposures

For chronic full life cycle studies, six adult rivulus (2 from each clone) were randomly (random number table) selected for each of three sublethal concentrations for each of the five chemical toxicants (90 fish). Also, twelve were randomly chosen for the uncontaminated water control and six for the acetone contaminated control (8.4 mg/ℓ acetone). Acetone was used as a carrier and never exceeded 8.4 mg/ℓ. Except for bromoform exposed fish all were held singly in reproduction chambers (see Section 2) and solutions (contaminated and control) were made fresh and changed weekly. Bromoform exposed fish were held singly in air-tight reproduction chambers fashioned from specimen jars. Bromoform solutions were made fresh and changed every time the air-tight containers were opened for feeding or egg collection (3 times per week). Offspring held at the same exposure levels as parental adults were treated the same.

Parental adults were exposed from 20 October 1978 until 20 June 1979. Eggs were collected three times a week and were randomly assigned to uncontaminated culture water or to culture water of the same type and degree of contamination as that of parental adults. This was done primarily to allow distinction between congenital effects (teratogenesis) and post-hatching developmental effects or other chronic effects such as pathologic changes resulting from direct sublethal exposure. On 20 September 1979 the experiment was terminated and all offspring were preserved in either 10%

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seawater buffered formalin or Bouins fixative (about half in each). Thus, the youngest offspring were 3 months old. After one week fixed fish were transferred permanently to ethyl alcohol (70%) after several changes to wash out fixative. In retrospect, 10% formalin is the superior fixative for it allows histological procedures as well as clearing and staining procedures. Standard histological procedures were used. Paraffin sections were cut 6 μm thick and stained with hematoxylin and eosin (H&E). Five to ten $F_1$ fish were randomly chosen for histological analysis from each of the various contaminated and uncontaminated conditions. All remaining specimens fixed in formalin were cleared and stained by the method of Dingerkus and Uhler (1977). Any histopathologic changes and skeletal abnormalities were recorded. During the course of the chronic study, records were kept on adult, larval and embryo mortalities and on gross abnormalities. All embryos were staged and any developmental abnormalities were described and recorded.

## RESULTS

A summary of the static 96-hr LC50 data for *Fundulus heteroclitus* postlarvae is presented in Table 7. Pentachlorophenol was most toxic followed by tetrachlorophenol, trichlorophenol and dibutyl phthalate, respectively. Bromoform was least toxic and had a narcotic effect on postlarvae. For this reason the LC50 was difficult to determine. Narcotized fish transferred from bromoform solution to uncontaminated water would often revive.

A summary of the static 96-hr LC50 data for *Rivulus marmoratus* postlarvae is given in Table 8. The same order of toxicity occurred; however, in all cases rivulus LC50's were lower than *F. heteroclitus* LC50's. This suggests that rivulus is more sensitive to the toxic effects of these chemicals; however, the difference is not statistically significant ($p > .05$). Thus, the LC50's for rivulus corroborate those for *F. heteroclitus*.

It is apparent from Table 8 that the response of rivulus postlarvae is highly uniform; that is, groups of individuals behave as if they were a single individual and genetically they are. A series of concentrations must be very tight in order to observe percent responses other than 0 and 100. In other words, probit lines are nearly vertical. This is a desirable characteristic because precision is high.

Tables 9 through 14 summarize the results of chronic exposures of rivulus: controls, pentachlorophenol (PCP), 2,3,4,6-tetrachlorophenol (TECP), 2,3,5-trichlorophenol (TRCP), dibutyl phthalate (DBP) and bromoform (BRO) are given, respectively. The number of viable eggs produced was quite variable among the various exposures. This reflects both the variability of total egg production among individual fish and variability due to production of presumably infertile eggs. Production of infertile eggs varied from 0% to 70% among different individuals but overall averaged 22.6%. Another source of variability relates to the original design of the reproduction chambers which was faulty and allowed fish to eat a larger proportion of their eggs. The improved design (given in Section 2 of this report) eliminated this as a significant problem.

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Table 7. Static 96-Hr LC50 Data for Postlarvae of Fundulus heteroclitus and Prescribed Concentrations for Chronic Study with Rivulus marmoratus

| Chemical | LC50(mg/ℓ)[a,c] | N[b] | 95% Confidence Limits[a] | Chronic Exposure Levels (mg/ℓ) |
|---|---|---|---|---|
| Pentachlorophenol | 0.370 | 50 | 0.195 - 0.535 | 0.074, 0.037, 0.0185 |
| 2,3,4,6-Tetrachlorophenol | 1.10 | 50 | 0.540 - 1.87 | 0.220, 0.110. 0.055 |
| 2,3,5-Trichlorophenol | 1.80 | 50 | 1.41 - 2.40 | 0.360, 0.180, 0.090 |
| Dibutyl Phthalate | 3.70 | 50 | 1.50 - 4.73 | 0.740, 0.370, 0.185 |
| Bromoform | 42.0 | 30 | 23.0 - 60.0 | 8.40, 4.20, 2.10 |

[a] Dose-mortality evaluated by probit analysis.

[b] Number of postlarvae per concentration; minimum of 5 concentrations per chemical.

[c] Acetone controls and uncontaminated controls (50 postlarvae each) had no mortalities.

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Table 8. Static 96-Hr LC50 Data for Postlarvae of *Rivulus marmoratus*

| Chemical | Concentration (mg/ℓ) | N | Mortality (%) | LC50[a] (mg/ℓ) |
|---|---|---|---|---|
| Pentachlorophenol | 0.70 | 10 | 100 | |
| | 0.50 | 10 | 100 | |
| | 0.35 | 10 | 100 | |
| | 0.25 | 10 | 70 | |
| | 0.24 | 10 | 10 | |
| | 0.22 | 10 | 0 | 0.245 |
| 2,3,4,6-Tetrachlorophenol | 1.44 | 10 | 100 | |
| | 1.00 | 10 | 100 | |
| | 0.91 | 10 | 100 | |
| | 0.84 | 10 | 100 | |
| | 0.79 | 10 | 90 | |
| | 0.70 | 10 | 0 | |
| | 0.50 | 10 | 0 | 0.76 |
| 2,3,5-Trichlorophenol | 2.6 | 10 | 100 | |
| | 1.8 | 10 | 100 | |
| | 1.7 | 10 | 100 | |
| | 1.6 | 10 | 20 | |
| | 1.5 | 10 | 10 | |
| | 1.3 | 10 | 0 | 1.62 |
| Dibutyl Phthalate | 5.0 | 8 | 100 | |
| | 3.7 | 8 | 75 | |
| | 2.7 | 8 | 0 | 3.6 |
| Bromoform[b] | 60.0 | 10 | 100 | |
| | 42.0 | 10 | 100 | |
| | 30.0 | 10 | 100 | |
| | 21.0 | 10 | 0 | |
| | 15.0 | 10 | 0 | |
| | 11.0 | 10 | 0 | 25.0 |
| Control - 1 | 0.0 | 10 | 0 | |
| Control - 2 | Acetone 2.6 | 10 | 0 | |

[a] LC50 determined graphically

[b] Test run in air-tight containers

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Table 9. Summary of Mortality and Skeletal Abnormality Data for *Rivulus marmoratus* Used as Controls for Chronic Exposure Study

| | Nominal Water Concentration (mg/ℓ) | | |
|---|---|---|---|
| | 0.00 | Acetone 8.4 | 8.4 Acetone-C[c] |
| Number of Adults (20 Oct 1978) | 12 | 6 | |
| Number of Adults (20 June 1979)[a] | 10 | 6 | |
| Number of Viable Eggs[b] | 250 | 172 | |
| Number Hatched | 167 | 131 | |
| Hatch (%) | 66.8 | 76.2 | |
| Number of Offspring[d] | 143 | 44 | 87 |
| Number of Mortalities | 42 | 18 | 42 |
| Mortality (%) | 29.4 | 40.9 | 48.3 |
| | Abnormalities | | |
| Number of Skeletal Abnormalities per Fish Examined | 14/47 | 2/10 | 1/8 |
| Skeletal Abnormalities (%) | 29.8 | 20.0 | 12.5 |

[a] Parental adults were reared from 20 October 1978 through 20 June 1979 but offspring were reared until 20 September 1979.

[b] Eggs were judged viable or non-viable (unfertilized?) at time of collection.

[c] Parental adults were exposed to concentrations indicated but offspring were reared in uncontaminated water.

[d] Offspring not accounted for were damaged while handling, used for histology or lost while changing solutions.

Table 10. Summary of Mortality and Skeletal Abnormality Data for *Rivulus marmoratus* from Chronic Exposure to Pentachlorophenol (PCP)

| | Nominal Water Concentrations (mg/ℓ) | | | | | |
|---|---|---|---|---|---|---|
| | 0.074 | | 0.037 | | 0.0185 | |
| Number of Adults (20 Oct 1978) | 6 | | 6 | | 6 | |
| Number of Adults (20 June 1979)[a] | 5 | | 4 | | 6 | |
| Number of Viable Eggs[b] | 160 | | 128 | | 234 | |
| Number Hatched | 138 | | 112 | | 186 | |
| Hatch (%) | 86.3 | | 87.5 | | 79.5 | |
| | | 0.074 PCP-C[c] | | 0.037 PCP-C[c] | | 0.0185 PCP-C[c] |
| Number of Offspring[d] | 57 | 54 | 36 | 34 | 103 | 67 |
| Number of Mortalities | 34 | 15 | 10 | 21 | 45 | 40 |
| Mortality (%) | 59.6 | 27.8 | 37.8 | 61.8 | 43.7 | 59.7 |
| | Abnormalities | | | | | |
| Number of Skeletal Abnormalities per Fish Examined | 4/11 | 8/14 | 4/17 | 3/8 | 20/33 | 10/15 |
| Skeletal Abnormalities (%) | 36.4 | 57.1 | 23.5 | 37.5 | 60.6 | 66.7 |

[a] Parental adults were exposed from 20 October 1978 through 20 June 1979 but offspring were exposed until 20 September 1979.

[b] Eggs were judged viable or non-viable (unfertilized?) at time of collection.

[c] Parental adults exposed to concentrations indicated but offspring reared in uncontaminated water.

[d] Offspring not accounted for were damaged while handling, used for histology or lost while changing solutions.

Table 11. Summary of Mortality and Skeletal Abnormality Data for *Rivulus marmoratus* from Chronic Exposure to 2,3,4,6-Tetrachlorophenol(TECP)

| | Nominal Water Concentration (mg/ℓ) | | | | | |
|---|---|---|---|---|---|---|
| | 0.220 | | 0.110 | | 0.055 | |
| Number of Adults (20 Oct 1978) | 6 | | 6 | | 6 | |
| Number of Adults (20 June 1979)[a] | 5 | | 5 | | 5 | |
| Number of Viable Eggs[b] | 119 | | 261 | | 203 | |
| Number Hatched | 99 | | 211 | | 166 | |
| Hatch (%) | 83.2 | | 80.8 | | 81.8 | |
| | | 0.220 TECP-C [c] | | 0.110 TECP-C [c] | | 0.055 TECP-C [c] |
| Number of Offspring[d] | 52 | 29 | 101 | 81 | 62 | 74 |
| Number of Mortalities | 33 | 8 | 58 | 29 | 20 | 34 |
| Mortality (%) | 63.5 | 27.6 | 57.4 | 35.8 | 32.3 | 45.9 |
| | Abnormalities | | | | | |
| Number of Skeletal Abnormalities Per Fish Examined | 7/8 | 7/14 | 16/21 | 13/28 | 10/20 | 4/20 |
| Skeletal Abnormalities[e] (%) | 87.5 | 50.0 | 76.2 | 46.4 | 50.0 | 20.0 |

[a] Parental adults were exposed from 20 October 1978 through 20 June 1979 but offspring were exposed until 20 September 1979.

[b] Eggs were judged viable or non-viable (unfertilized?) at time of collection.

[c] Parental adults were exposed to concentrations indicated but these offspring were reared in uncontaminated water.

[d] Offspring not accounted for were damaged while handling, used for histology or lost while changing solutions.

[e] Fin erosion included under skeletal anomalies.

Table 12. Summary of Mortality and Skeletal Abnormality Data for *Rivulus marmoratus* from Chronic Exposure to 2,3,5-Trichlorophenol (TRCP)

| | Nominal Water Concentration (mg/ℓ) | | | | | |
|---|---|---|---|---|---|---|
| | 0.36 | | 0.18 | | 0.09 | |
| Number of Adults (20 Oct 1978) | 6 | | 6 | | 6 | |
| Number of Adults (20 June 1979)[a] | 3 | | 5 | | 6 | |
| Number of Viable Eggs[b] | 105 | | 204 | | 196 | |
| Number Hatched | 92 | | 183 | | 184 | |
| Hatch (%) | 87.6 | | 89.7 | | 93.9 | |
| | | 0.36 TRCP-C [c] | | 0.18 TRCP-C [c] | | 0.09 TRCP-C [c] |
| Number of Offspring[d] | 52 | 40 | 112 | 71 | 87 | 91 |
| Number of Mortalities | 8 | 7 | 18 | 8 | 22 | 23 |
| Mortality (%) | 15.4 | 17.5 | 16.1 | 11.3 | 25.3 | 25.3 |
| | Abnormalities | | | | | |
| Number of Skeletal Abnormalities per Fish Examined | 8/21 | 5/15 | 15/79 | 8/29 | 12/54 | 11/33 |
| Skeletal Abnormalities (%) | 38.1 | 33.3 | 19.0 | 27.6 | 22.2 | 33.3 |

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[a] Parental adults were exposed from 20 October 1978 through 20 June 1979 but offspring were exposed until 20 September 1979.

[b] Eggs were judged viable or non-viable (unfertilized?) at time of collection.

[c] Parental adults were exposed to concentrations indicated but these offspring were reared in uncontaminated water.

[d] Offspring not accounted for were damaged while handling, used for histology or lost while changing solutions.

Table 13. Summary of Mortality and Skeletal Abnormality Data for *Rivulus marmoratus* from Chronic Exposure to Dibutyl Phthalate (DBP)

| | Nominal Water Concentrations (mg/ℓ) | | | | | |
|---|---|---|---|---|---|---|
| | 0.740 | 0.740 DBP-C [c] | 0.370 | 0.370 DBP-C [c] | 0.185 | 0.185 DBP-C [c] |
| Number of Adults (20 Oct 1978) | 6 | | 6 | | 6 | |
| Number of Adults (20 June 1979)[a] | 4 | | 5 | | 5 | |
| Number of Viable Eggs[b] | 186 | | 288 | | 222 | |
| Number Hatched | 139 | | 240 | | 171 | |
| Hatch (%) | 74.7 | | 83.3 | | 77.0 | |
| Number of Offspring[d] | 74 | 47 | 114 | 96 | 90 | 55 |
| Number of Mortalities | 38 | 30 | 35 | 41 | 51 | 31 |
| Mortality (%) | 51.4 | 63.8 | 30.7 | 42.7 | 56.7 | 56.4 |
| | Abnormalities | | | | | |
| Number of Skeletal Abnormalities per Fish Examined | 7/10 | 7/9 | 32/60 | 19/34 | 10/20 | 3/13 |
| Skeletal Abnormalities (%) | 70.0 | 77.7 | 53.3 | 55.9 | 50.0 | 23.1 |

[a] Parental adults were exposed from 20 October 1978 through 20 June 1979 but offspring were exposed until 20 September 1979.

[b] Eggs were judged viable or non-viable (unfertilized?) at time of collection.

[c] Parental adults exposed to concentrations indicated but offspring reared in uncontaminated water.

[d] Offspring not accounted for were damaged while handling, used for histology or lost while changing solutions.

Table 14. Summary of Mortality and Skeletal Abnormality Data for *Rivulus marmoratus* from Chronic Exposure to Bromoform(BRO)

| | Nominal Water Concentration (mg/ℓ) | | | | | |
|---|---|---|---|---|---|---|
| | 8.4 | | 4.2 | | 2.1 | |
| Number of Adults (20 Oct 1978) | 6 | | 6 | | 6 | |
| Number of Adults (20 June 1979)[a] | 6 | | 5 | | 4 | |
| Number of Viable Eggs[b] | 250 | | 169 | | 238 | |
| Number Hatched | 197 | | 109 | | 171 | |
| Hatch (%) | 78.8 | | 64.5 | | 71.8 | |
| | | 8.4 BRO-C[c] | | 4.2 BRO-C[c] | | 2.1 BRO-C[c] |
| Number of Offspring[d] | 88 | 100 | 45 | 60 | 68 | 101 |
| Number of Mortalities | 26 | 23 | 19 | 15 | 34 | 38 |
| Mortality (%) | 29.5 | 23.0 | 42.2 | 25.0 | 50.0 | 37.6 |
| | Abnormalities | | | | | |
| Number of Skeletal Abnormalities per Fish Examined | 9/24 | 11/33 | 12/34 | 5/19 | 7/14 | 12/27 |
| Skeletal Abnormalities (%) | 37.5 | 33.3 | 35.3 | 26.3 | 50.0 | 44.4 |

[a] Parental adults were exposed from 20 October 1978 through 20 June 1979 but offspring were exposed until 20 September 1979.

[b] Eggs were judged viable or non-viable (unfertilized?) at time of collection.

[c] Parental adults exposed to concentrations indicated but offspring reared in uncontaminated water.

[d] Offspring not accounted for were damaged while handling, used for histology or lost while changing solutions.

The percent of viable eggs that hatched varied from about 70 to 90% overall and was toward the lower end of this range in controls.

By far the dominant abnormality among embryos consisted of, or was related to, an edematous pericardial cavity (Fig. 12). In its most exaggerated form the pericardium ballooned around the head and often contained a flocculent material of unknown composition. Also the heart appeared string-like and was inefficient in pumping blood. Less exaggerated examples had a slight bulge in the pericardium with no discernable alteration in the heart. This abnormality always resulted in abnormal growth and development of the embryo. Extreme cases never hatched whereas moderately affected embryos were often stunted with curved or bent nonfunctional tails. There was no particular pattern of this expression apparent among the various chemical exposures or controls (Table 15); therefore, this abnormality did not result from the exposures. Other abnormalities included blastulae with irregular blastomeres, bubble-like expansions of the vitelline membrane and twinning (occurred once) but these were quite infrequent and showed no particular pattern among the various exposures or controls.

Mortality rates among post-hatch offspring were also variable. Mortality in controls varied from about 30 to 50% as did mortalities of offspring from the bromoform and dibutyl phthalate exposure. Mortality rates of offspring of some of the groups from the PCP and TECP exposures went as high as about 60%. In contrast the mortality rate of offspring from the TRCP exposures were consistently low (ca. 10-25%).

The frequency of skeletal abnormalities varied somewhat over the various exposure series; however, certain trends are evident. Rates of skeletal abnormality in controls were about 30%. Clear dose-response rates were evident in offspring of TECP and DBP exposures.

Skeletal abnormalities observed in cleared and stained fish were divided into six categories (Table 16 to 25): vertebral fusion, deformed centra, abnormal neural and/or hemal spines, abnormal dorsal fins, abnormal pectoral fins, and abnormal pelvic fins. Vertebral fusion is the joining of adjacent centra into a solid immovable structure without discernable articulation faces (compare Figs. 13a and 13b). Deformed centra or kinks appear to result from the fusion of adjacent neural or hemal spines, arches or zygapophyses. The articulation faces of the vertebrae are thus held askew and a sharp bend or kink in the spinal column results (Figs. 14a-c). Neural and hemal spine deformities include bilateral splits, fusions, disorientations or total lack of spines (see Figs. 14a-c and 15a, b). In cleared and stained specimens the distinction between abnormal fins and fin erosion is clear. Parts or all of the supporting skeleton are lacking in deformed fins whereas eroded fins lack parts or all of the fin rays. The erosion process generally proceeds from the tips of the rays toward the base (Figs. 16a-c). Pterygiophores of abnormal dorsal fins may be lacking, disoriented or fused (Figs. 17a-c). Abnormal pectoral fins generally have a withered appearance with few rays. Also, supporting radials, scapula and the dorsal portion of the coracoid bones may be partially or completely absent (Figs. 18a, b). Pelvic fins develop last among fins, approximately two months after hatching,

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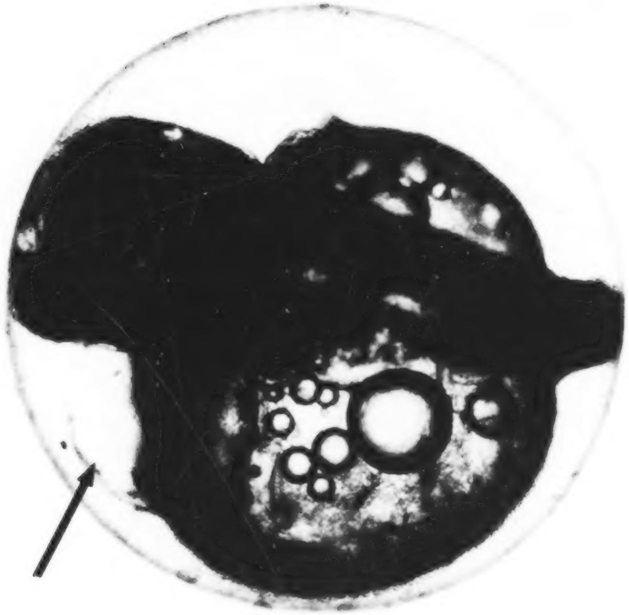

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Figure 12. Photomicrograph of a late stage embryo of rivulus. Note edematous pericardial cavity (arrow).

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Table 15. Distribution of Heart Abnormalities in Embryos of *Rivulus marmoratus* Exposed Chronically to Various Toxicants

| Chemical | Concentration (mg/ℓ) | Heart Abnormalities (%)[a] |
|---|---|---|
| Pentachlorophenol | 0.074 | 6.25 |
| | 0.037 | 2.34 |
| | 0.0185 | 9.40 |
| 2,3,4,6-Tetrachlorophenol | 0.22 | 2.52 |
| | 0.11 | 3.45 |
| | 0.05 | 7.88 |
| 2,3,5-Trichlorophenol | 0.36 | 0.00 |
| | 0.18 | 0.98 |
| | 0.09 | 2.04 |
| Dibutyl phthalate | 0.74 | 6.45 |
| | 0.37 | 5.90 |
| | 0.185 | 8.56 |
| Bromoform | 8.4 | 1.20 |
| | 4.2 | 4.14 |
| | 2.1 | 2.52 |
| Control | 0.00 | 6.80 |
| Acetone | 8.40 | 2.91 |

[a] Determined at time of egg collection; consisted of expanded pericardial cavity with string-like heart in varying degrees.

Table 16. Distribution of Skeletal Abnormalities of Offspring of *Rivulus marmoratus* Exposed to Pentachlorophenol.[a]

| | Nominal Water Concentrations (mg/ℓ) | | | |
|---|---|---|---|---|
| | 0.0740 | 0.0370 | 0.0185 | 0.00[b] |
| Vertebral Fusion | 1/11(9.1)[c] | 3/17(17.6) | 9/31(29.0) | 5/65(7.70 |
| Deformed Centra (kink) | 0/11(0) | 0/17(0) | 7/31(22.6) | 6/65(9.2) |
| Abnormal Neural and/or Hemal Spines | 2/11(18.2) | 3/17(17.6) | 13/31(41.9) | 9/65(13.8) |
| Abnormal Dorsal Fin | 0/11(0) | 0/17(0) | 0/31(0) | 0/65(0) |
| Abnormal Pectoral Fins | 0/11(0) | 0/17(0) | 4/31(12.9) | 2/65(3.1) |
| Abnormal Pelvic Fins | 0/11(0) | 0/17(0) | 4/31(12.9) | 2/65(3.1) |
| Erosion of Fin Rays | 0/11(0) | 0/17(0) | 0/31(0) | 0/65(0) |
| Skeletal Abnormalities (Total) | 2/11(18.2) | 3/17(17.6) | 20/31(64.5) | 17/65(26.2) |

[a] Parental adults and offspring reared in indicated concentrations of PCP.

[b] Clean water controls and acetone controls combined.

[c] Nos. in parentheses are percents.

Table 17. Distribution of Skeletal Abnormalities of Offspring of *Rivulus marmoratus*; Parental Adults Only were Exposed to Pentachlorophenol[a].

| | Nominal Water Concentrations (mg/ℓ) | | | |
|---|---|---|---|---|
| | 0.0740 | 0.0370 | 0.0185 | 0.00[b] |
| Vertebral Fusion | 3/14(21.4)[c] | 0/8(0) | 8/15(53.3) | 5/65(7.7) |
| Deformed Centra (kink) | 3/14(21.4) | 1/8(12.5) | 1/15(6.7) | 6/65(9.2) |
| Abnormal Neural and/or hemal spines | 5/14(35.7) | 3/8(37.5) | 5/15(33.3) | 9/65(13.8) |
| Abnormal Dorsal Fins | 0/14(0) | 0/8(0) | 0/15(0) | 0/65(0) |
| Abnormal Pectoral Fins | 0/14(0) | 0/8(0) | 0/15(0) | 2/65(3.1) |
| Abnormal Pelvic Fins | 0/14(0) | 0/8(0) | 0/15(0) | 2/65(3.1) |
| Erosion of Fin Rays | 0/14(0) | 0/8(0) | 0/15(0) | 0/65(0) |
| Skeletal Abnormalities (Total) | 7/14(50.0) | 3/8(37.5) | 8/15(53.3) | 17/65(26.2) |

[a] Parental adults were held in indicated concentrations of PCP but offspring were reared in uncontaminated water.

[b] Clean water controls and acetone controls combined.

[c] Nos. in parentheses are percents.

Table 18. Distribution of Skeletal Abnormalities of Offspring of *Rivulus marmoratus* Exposed to 2,3,4,6-Tetrachlorophenol.[a]

| | Nominal Water Concentrations (mg/ℓ) | | | |
|---|---|---|---|---|
| | 0/220 | 0.110 | 0.055 | 0.00[b] |
| Vertebral Fusion | 1/8(12.5)[c] | 10/21(47.6) | 5/20(25.0) | 5/65(7.7) |
| Deformed Centra (kink) | 0/8(0) | 1/21(4.8) | 2/20(10.0) | 6/65(9.2) |
| Abnormal Neural and/or Hemal Spines | 2/8(25.0) | 12/21(57.1) | 5/20(25.0) | 9/65(13.8) |
| Abnormal Dorsal Fins | 0/8(0) | 0/21(0) | 1/20(5.0) | 0/65(0) |
| Abnormal Pectoral Fins | 1/8(12.5) | 5/21(23.8) | 1/20(5.0) | 2/65(3.1) |
| Abnormal Pelvic Fins | 0/8(0) | 1/21(4.8) | 0/20(0) | 0/65(3.1) |
| Erosion of Fin Rays | 5/8(62.5) | 10/21(47.6) | 6/20(30.0 | 0/65(0) |
| Skeletal Abnormalities (Total) | 5/8(62.5) | 16/21(76.2) | 10/20(50.0) | 17/65(26.2) |

[a] Parental adults and offspring reared in indicated concentrations of TECP.

[b] Clean water controls and acetone controls combined.

[c] Nos. in parentheses are percents.

Table 19. Distribution of Skeletal Abnormalities of Offspring of *Rivulus marmoratus*; Parental Adults Only were Exposed to 2,3,4,6-Tetrachlorophenol.[a]

| | Nominal Water Concentrations (mg/ℓ) | | | |
|---|---|---|---|---|
| | 0.220 | 0.110 | 0.055 | 0.00[b] |
| Vertebral Fusion | 6/14(42.9)[c] | 9/28(32.1) | 3/20(15.0) | 5/65(7.7) |
| Deformed Centra (kink) | 0/14(0) | 1/28(3.6) | 0/20(0) | 6/65(9.2) |
| Abnormal Neural and/or Hemal Spines | 4/14(28.6) | 9/28(32.1) | 3/20(15.0) | 9/65(13.8) |
| Abnormal Dorsal Fins | 1/14(7.1) | 0/28(0) | 1/20(5.0) | 0/65(0) |
| Abnormal Pectoral Fins | 0/14(0) | 3/28(10.7) | 0/20(0) | 2/65(3.1) |
| Abnormal Pelvic Fins | 0/14(0) | 1/28(3.6) | 0/20(0) | 2/65(3.1) |
| Erosion of Fin Rays | 0/14(0) | 0/28(0) | 0/20(0) | 0/65(0) |
| Skeletal Abnormalities (Total) | 7/14(50.0) | 13/28(46.4) | 4/20(20.0) | 17/65(26.2) |

[a] Parental adults were held in indicated concentrations of TECP but offspring were reared in uncontaminated water.

[b] Clean water controls and acetone controls combined.

[c] Nos. in parentheses are percents.

Table 20. Distribution of Skeletal Abnormalities of Offspring of *Rivulus marmoratus* Exposed to 2,3,5-Trichlorophenol.[a]

| | Nominal Water Concentration (mg/ℓ) | | | |
|---|---|---|---|---|
| | 0.360 | 0.180 | 0.090 | 0.00[b] |
| Vertebral Fusion | 3/21(14.3)[c] | /79(3.8) | 6/54(11.1) | 5/65(7.7) |
| Deformed Centra (kink) | 1/21(4.8) | 1/79(1.3) | 0/54(0) | 6/65(9.2) |
| Abnormal Neural and/or Hemal Spines | 5/21(23.8) | 15/79(19.0) | 9/54(16.7) | 9/65(13.8) |
| Abnormal Dorsal Fins | 0/21(0) | 0/79(0) | 0/54(0) | 0/65(0) |
| Abnormal Pectoral Fins | 0/21(0) | 0/79(0) | 0/54(0) | 2/65(3.1) |
| Abnormal Pelvic Fins | 3/21(14.3) | 1/79(1.3) | 0/54(0) | 2/65(3.1) |
| Erosion of Fin Rays | 0/21(0) | 0/79(0) | 0/54(0) | 0/65(0) |
| Skeletal Abnormalities (Total) | 8/21(38.1) | 15/79(19.0) | 12/54(22.2) | 17/65(26.2) |

[a] Parental adults and offspring reared in indicated concentrations of TRCP.

[b] Clean water controls and acetone controls combined.

[c] Nos. in parentheses are percents.

Table 21. Distribution of Skeletal Abnormalities of Offspring of *Rivulus marmoratus*; Parental Adults Only were Exposed to 2,3,5-Trichlorophenol.[a]

| | Nominal Water Concentration (mg/ℓ) | | | |
|---|---|---|---|---|
| | 0.360 | 0.180 | 0.090 | 0.00[b] |
| Vertebral Fusion | 3/15(20.0)[c] | 7/30(23.3) | 5/33(15.2) | 5/65(7.7) |
| Deformed Centra (kink) | 1/15(6.7) | 0/30(0) | 4/33(12.1) | 6/65(9.2) |
| Abnormal Neural and/or Hemal Spines | 0/15(0) | 7/30(23.3) | 7/33(21.2) | 9/65(13.8) |
| Abnormal Dorsal Fins | 0/15(0) | 0/30(0) | 0/33(0) | 0/65(0) |
| Abnormal Pectoral Fins | 2/15(13.3) | 2/30(6.7) | 1/33(3.0) | 2/65(3.1) |
| Abnormal Pelvic Fins | 0/15(0) | 0/30(0) | 0/33(0) | 2/65(3.1) |
| Erosion of Fin Rays | 0/15(0) | 0/30(0) | 0/33(0) | 0/65(0) |
| Skeletal Abnormalities (Total) | 5/15(33.3) | 9/30(30.0) | 11/33(33.3) | 17/65(26.2) |

[a] Parental adults were held in indicated concentrations of TRCP but offsprine were reared in uncontaminated water.

[b] Clean water controls and acetone controls combined.

[c] Nos. in parentheses are percents.

Table 22. Distribution of Skeletal Abnormalities of Offspring of *Rivulus marmoratus* Exposed to Dibutyl Phthalate.[a]

| | Nominal Water Concentration (mg/ℓ) | | | |
|---|---|---|---|---|
| | 0.740 | 0.370 | 0.185 | 0.00[b] |
| Vertebral Fusion | 6/17(35.3)[c] | 15/60(25.0) | 5/20(25.0) | 5/65(7.7) |
| Deformed Centra (kink) | 1/17(5.9) | 10/60(16.7) | 1/20(5.0) | 6/65(9.2) |
| Abnormal Neural and/or Hemal Spines | 4/17(23.5) | 13/60(21.7) | 9/20(45.0) | 9/65(13.8) |
| Abnormal Dorsal Fins | 0/17(0) | 2/60(3.3) | 0/20(0) | 0/65(0) |
| Abnormal Pectoral Fins | 1/17(5.9) | 2/60(3.3) | 2/20(10.0) | 2/65(3.1) |
| Abnormal Pelvic Fins | 0/17(0) | 0/60(0) | 0/20(0) | 2/65(3.1) |
| Erosion of Fin Rays | 0/17(0) | 0/60(0) | 0/20(0) | 0/65(0) |
| Skeletal Abnormalities (Total) | 10/17(58.8) | 32/60(53.3) | 10/20(50.0) | 17/65(26.2) |

[a] Parental adults and offspring reared in indicated concentrations of DBP.

[b] Clean water controls and acetone controls combined.

[c] Nos. in parentheses are percents.

Table 23. Distribution of Skeletal Abnormalities of Offspring of *Rivulus marmoratus*; Parental Adults Only were Exposed to Dibutyl Phthalate.[a]

| | Nominal Water Concentration (mg/ℓ) | | | |
|---|---|---|---|---|
| | 0.740 | 0.370 | 0.185 | 0.00[b] |
| Vertebral Fusion | 5/9(55.5)[c] | 12/34(35.5) | 3/13(23.1) | 5/65(7.7) |
| Deformed Centra (kink) | 0/9(0) | 1/34(2.9) | 0/13(0) | 6/65(9.2) |
| Abnormal Neural and/or Hemal Spines | 3/9(33.3) | 10/34(29.4) | 2/13(15.4) | 9/65(13.8) |
| Abnormal Dorsal Fin | 0/9(0) | 0/34(0) | 0/13(0) | 0/65(0) |
| Abnormal Pectoral Fins | 1/9(11.1) | 4/34(11.8) | 1/13(7.8) | 2/65(3.1) |
| Abnormal Pelvic Fins | 0/9(0) | 2/34(5.9) | 0/13(0) | 2/65(3.1) |
| Erosion of Fin Rays | 0/9(0) | 0/34(0) | 0/13(0) | 0/65(0) |
| Skeletal Abnormalities (Total) | 7/9(77.8) | 19/34(55.9) | 3/13(23.1) | 17/65(26.2) |

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[a] Parental adults were held in indicated concentrations of DBP but offspring were reared in uncontaminated water.

[b] Clean water controls and acetone controls combined.

[c] Nos. in parentheses are percents.

Table 24. Distribution of Skeletal Abnormalities of Offspring of *Rivulus marmoratus* Exposed to Bromoform.[a]

| | Nominal Water Concentration (mg/ℓ) | | | |
|---|---|---|---|---|
| | 8.40 | 4.20 | 2.10 | 0.00[b] |
| Vertebral Fusion | 2/24(8.3)[c] | 3/34(8.8) | 3/14(21.4) | 5/65(7.7) |
| Deformed Centra (kink) | 0/24(0) | 1/34(2.9) | 0/14(0) | 6/65(9.2) |
| Abnormal Neural and/or Hemal Spines | 4/24(16.7) | 3/34(8.8) | 4/14(28.6) | 9/65(13.8) |
| Abnormal Dorsal Fin | 4/24(16.7) | 3/34(8.8) | 2/14(14.3) | 0/65(0) |
| Abnormal Pectoral Fins | 1/24(4.2) | 1/34(2.9) | 0/14(0) | 2/65(3.1) |
| Abnormal Pelvic Fins | 1/24(4.2) | 6/34(17.6) | 0/14(0) | 2/65(3.1) |
| Erosion of Fin Rays | 0/24(0) | 0/34(0) | 0/14(0) | 0/65(0) |
| Skeletal Abnormalities (Total) | 9/24(37.5) | 12/34(35.3) | 7/14(50.0) | 17/65(26.2) |

[a] Parental adults and offspring reared in indicated concentration of bromoform.

[b] Clean water controls and acetone controls combined.

[c] Nos. in parentheses are percents.

Table 25. Distribution of Skeletal Abnormalities of Offspring of *Rivulus marmoratus*; Parental Adults Only were Exposed to Bromoform.[a]

| | Nominal Water Concentration (mg/ℓ) | | | |
|---|---|---|---|---|
| | 8.40 | 4.20 | 2.10 | 0.00[b] |
| Vertebral Fusion | 4/33(12.1)[c] | 1/20(5.0) | 7/27(25.9) | 5/65(7.7) |
| Deformed Centra (kink) | 0/33(0) | 1/20(5.0) | 1/27(3.7) | 6/65(9.2) |
| Abnormal Neural and/or Hemal Spines | 6/33(18.2) | 5/20(25.0) | 5/27(18.5) | 9/65(13.8) |
| Abnormal Dorsal Fin | 0/33(0) | 0/20(0) | 0/27(0) | 0/65(0) |
| Abnormal Pectoral Fins | 0/33(0) | 1/20(5.0) | 0/27(0) | 2/65(3.1) |
| Abnormal Pelvic Fins | 0/33(0) | 0/20(0) | 1/27(3.7) | 2/65(3.1) |
| Erosion of Fin Rays | 0/33(0) | 0/20(0) | 0/27(0) | 0/65(0) |
| Skeletal Abnormalities (Total) | 11/33(33.3) | 5/20(25.0) | 12/27(44.4) | 17/65(26.2) |

[a] Parental adults were held in indicated concentrations of bromoform but offspring were reared in uncontaminated water.

[b] Clean water controls and acetone controls combined.

[c] Nos. in parentheses are percents.

Figure 13 a,b. Photomicrograph of caudal skeletal of cleared and stained Rivulus marmoratus. a-Normal caudal skeletal from control fish. b-Specimen reared in uncontaminated water from parental adult exposed to 0.740 mg/ℓ dibutyl phthalate (DBP). Note fusion of four vertebrae (arrow) and deformed centrum of penultimate vertebra. Scale applies to a and b.

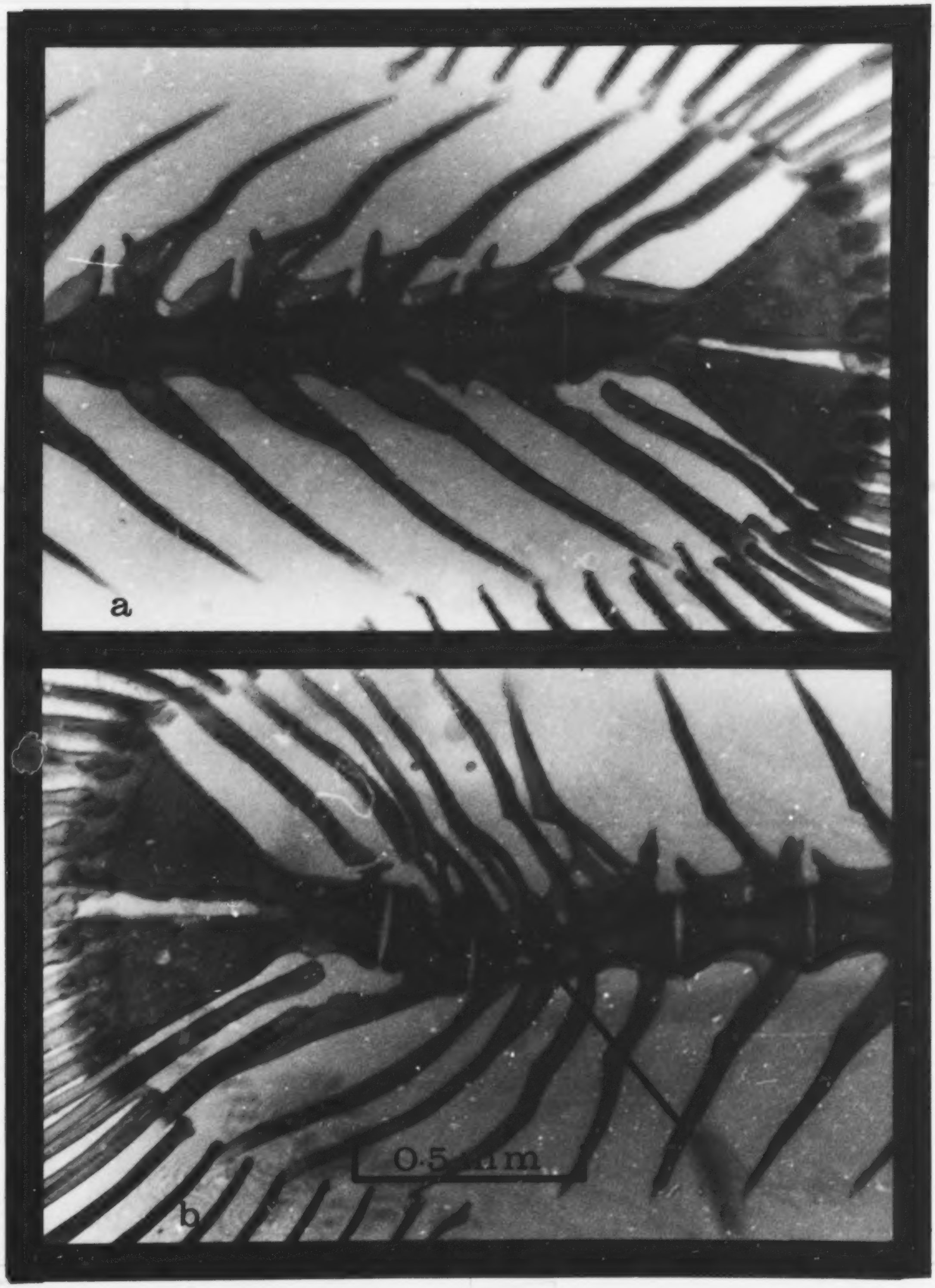
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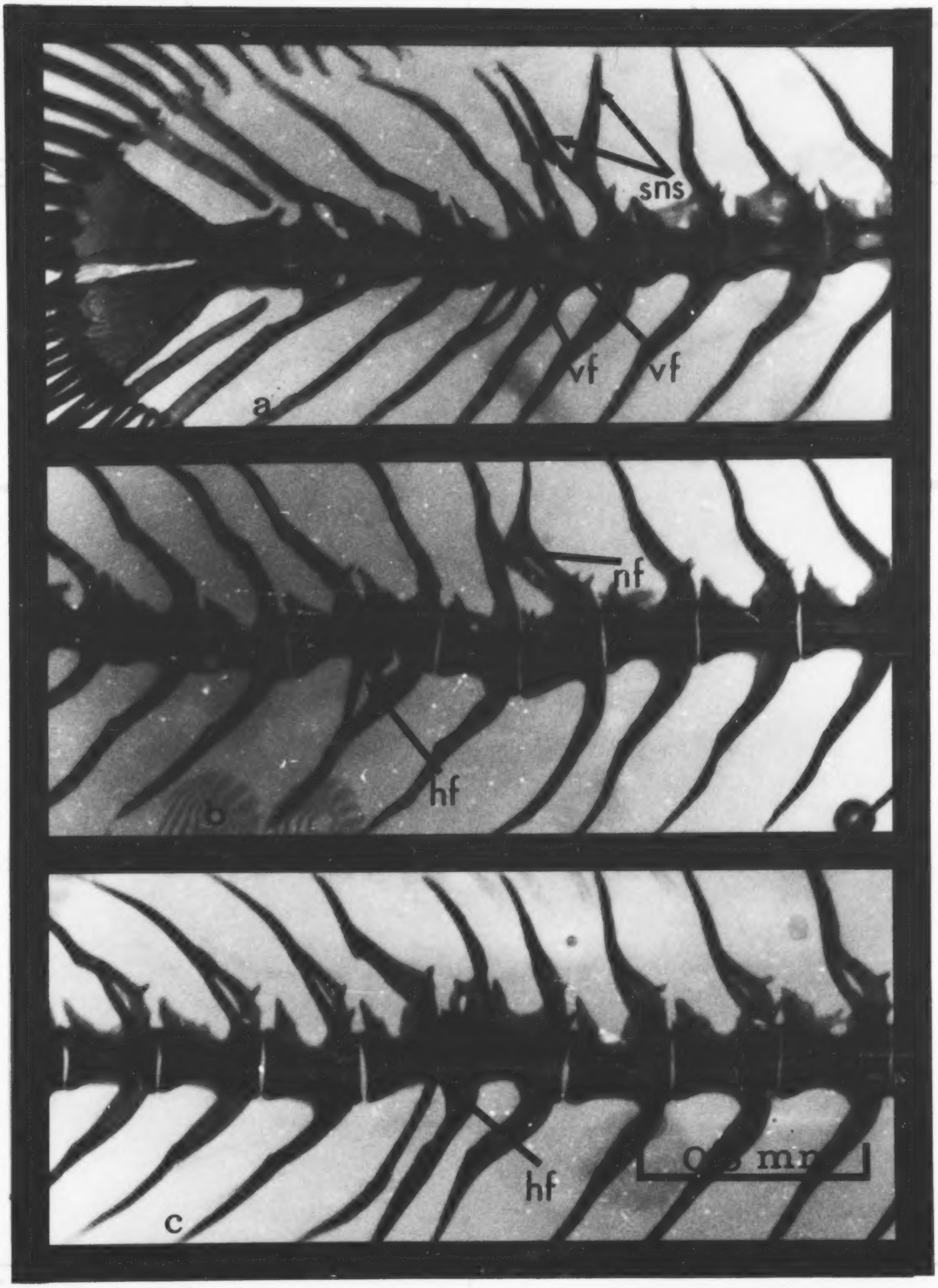
Figure 14a-c. Photomicrographs of caudal vertebrae of cleared and stained Rivulus marmoratus. a-Specimen (and parental adult) was continuously exposed to 0.370 mg/ℓ dibutyl phthalate (DBP) for entire life. Note medial split in neural spine (sns) and vertebral fusion (vf). b-Specimen (and parental adult) was continuously exposed to 0.370 mg/ℓ DBP for entire life. Note "kinked" vertebrae (deformed centra) held in position by fused neural spines (nf) and fused hemal spines (hf). c-Specimen reared in uncontaminated water from parental adult exposed to 0.740 mg/ℓ DBP. Note "kinked" vertebrae held in position by fused hemal spines (hf). Scale applies to a, b and c.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vf
vf
a
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0.5 mm
c

Figure 15a,b. Photomicrographs of trunk vertebrae of cleared and stained *Rivulus marmoratus*. a-Normal vertebrae of control fish. b-Specimen (and parental adult) was continuously exposed to 0.185 mg/ℓ dibutyl phthalate (DBP) for entire life. Note grossly abnormal neural spines and poorly formed hemal spines in exposed fish. Scale applies to a and b.

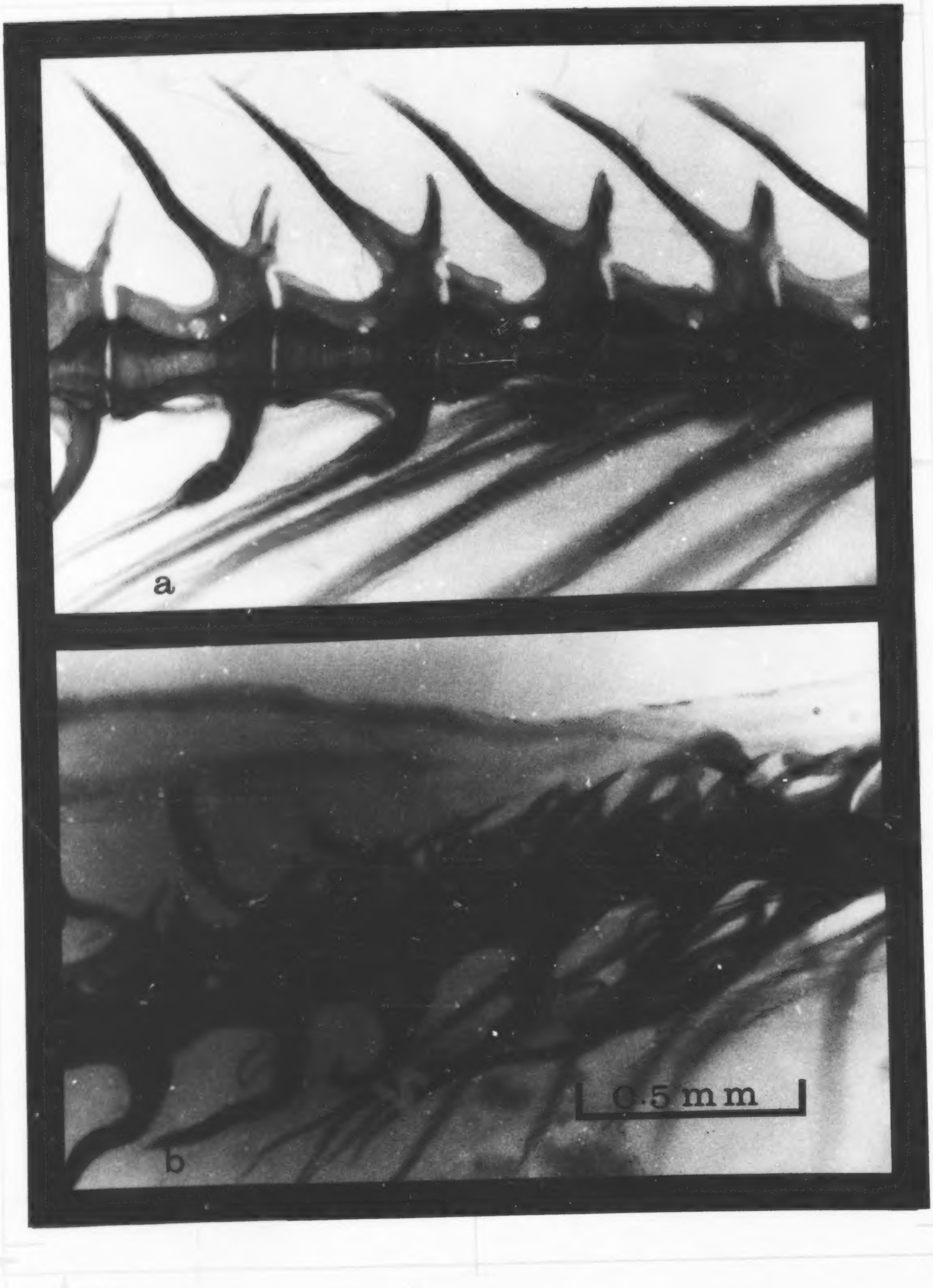
a
b
0.5 mm

Figure 16 a-c. Photomicrographs of cleared and stained Rivulus marmoratus. Specimens (and parental adults) were continuously exposed to the following concentrations of 2,3,4,6-tetrachlorophenol for entire life: a-0.055 mg/ℓ, b and c (same fish)-0.110 mg/ℓ. Note erosion of fin rays (arrows), also deformed neural and hemal spines and vertebral fusion ( a and b). Scale applies to a, b and c.

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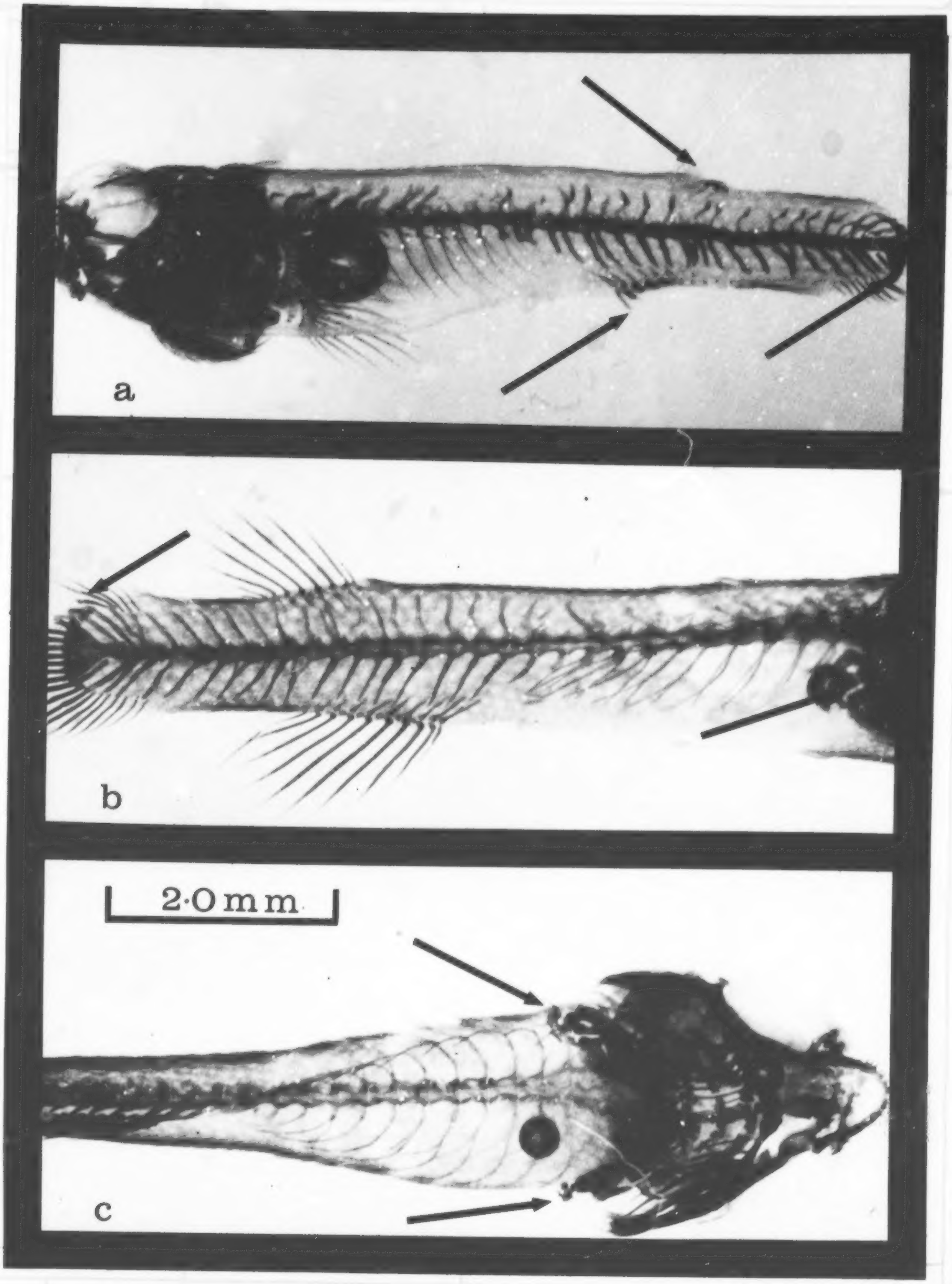

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Figure17a-c. Photomicrographs of cleared and stained _Rivulus marmoratus_. Specimens (and their parental adults) were continuously exposed to the following concentrations of bromoform for entire life: a-2.1 mg/ℓ, b-4.2 mg/ℓ, c-8.4 mg/ℓ. Note partial to complete loss of dorsal fin elements. a-proximal and distal pterygiophores (arrow) are in disarray and fin rays are absent. b-Pterygiophores (arrow) are reduced to two and fin rays are absent. c-All dorsal fin elements are lost, neural spines are deformed and some vertebrae are fused.

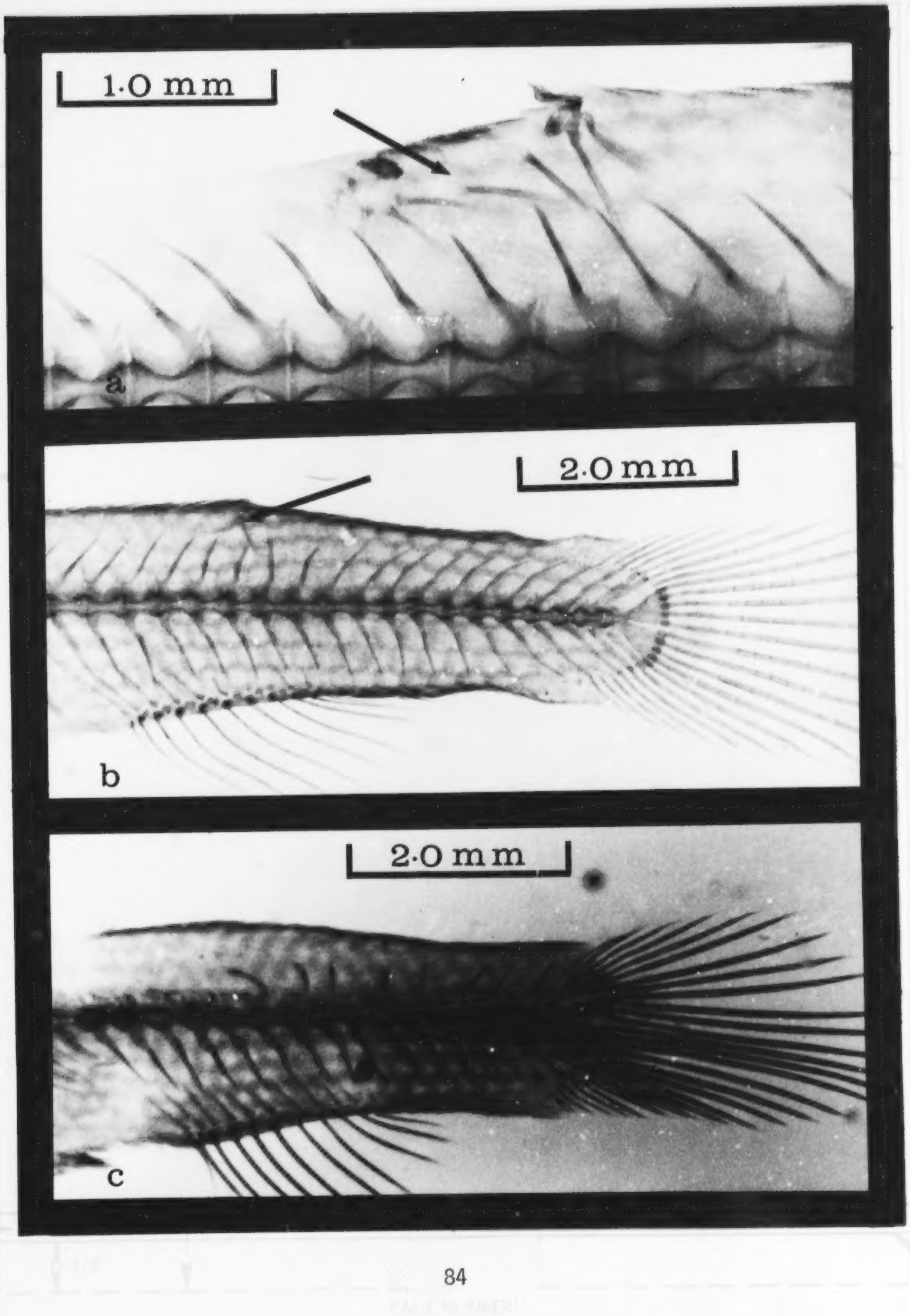
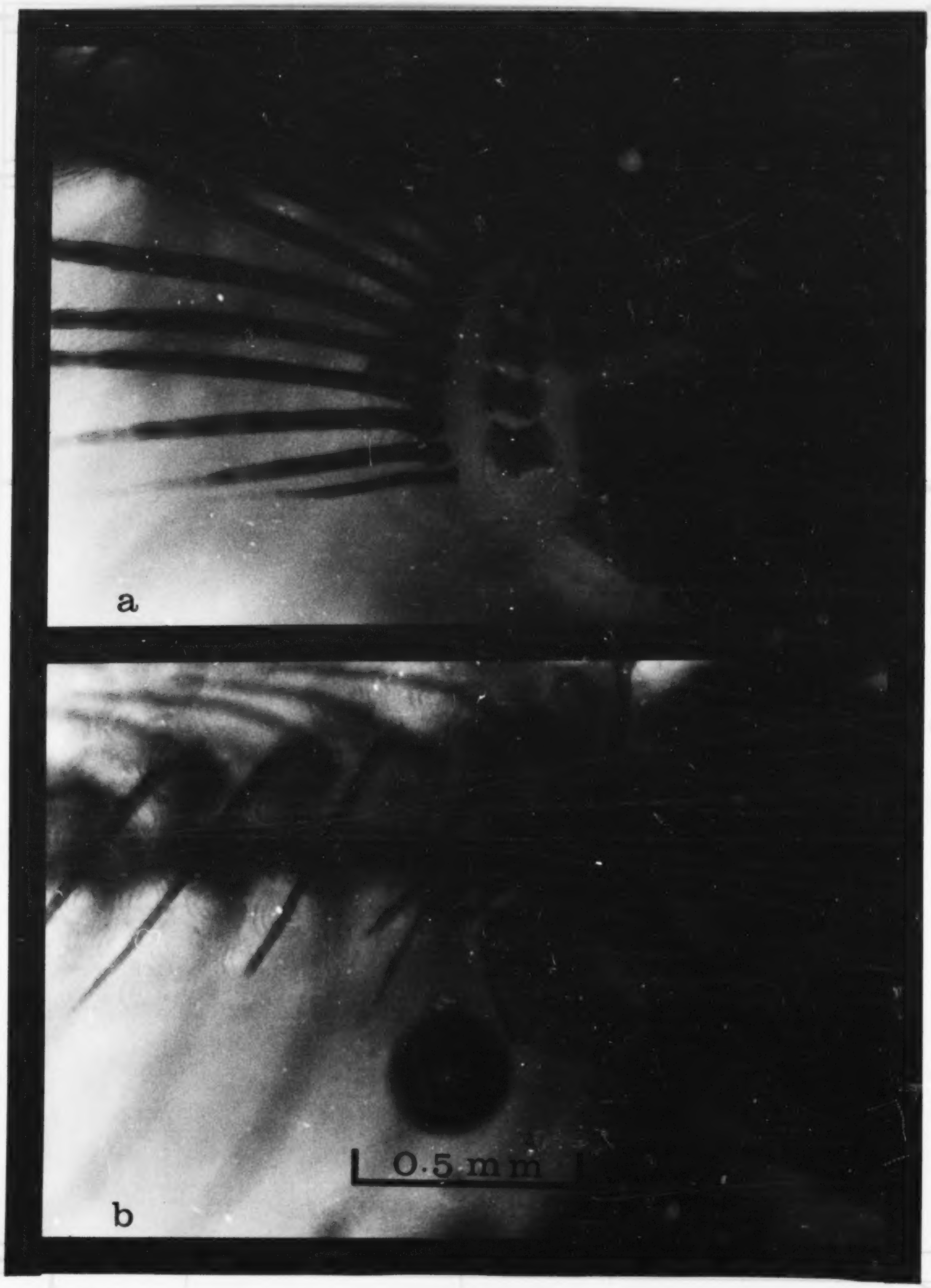

1.0 mm
a
2.0 mm
b
2.0 mm
c

Figure 18a, b. Photomicrographs of pectoral girdles of cleared and stained *Rivulus marmoratus*. a-Normal pectoral girdle of control fish. b-Specimen (and parental adult) was continuously exposed to 0.370 mg/ℓ dibutyl phthalate (DBP) for entire life. Note complete absence of fin rays and radials and partial loss of scapula and coracoid bones in exposed fish. Scale applies to a and b.


a
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b

under the culture conditions. Older specimens may lack one or both fins and their supporting girdles. Severely effected fish often showed multiple skeletal abnormalities (Figs. 19a-d).

The distribution of skeletal abnormalities in control as compared with PCP exposures (Tables 16 and 17) indicate no clear dose-response relationship. Although rates of abnormal neural and/or hemal spines were consistently high in offspring from PCP exposed fish reared in clean water (Table 17), low sample numbers preclude a clear relationship.

Erosion of fin rays was clearly the result of TECP exposure (Table 18). There was a clear dose-response relationship and fin ray erosion occurred in no other exposures or controls. That this response was the result of direct chronic exposure to TECP is demonstrated by the fact that offspring from TECP treated adults which were reared in clean water did not show the response (Table 19). Also, gill damage was evident in TECP treated offspring. In most severe cases there was loss of secondary lamellae and partial to nearly complete loss of gill filaments and a marked epithelial hyperplasia (compare Figs. 20 a & b). In less severe cases the terminal portions of the filaments were stunted and the lamellae were irregular in shape. Degree of gill damage showed a dose-response. Offspring of controls and offspring reared in clean water from TECP treated adults had normal gills.

There are also strong indications that vertebral fusion and abnormal neural and/or hemal spine rates follow a dose-response. This is especially evident in Table 19 (i.e., offspring reared in uncontaminated water) because rates of congenital abnormalities are not altered by the toxic effects of chronic exposure to TECP (see mortality rate in Table 11).

There are no clear dose-response relationships in the distributions of skeletal abnormalities in TRCP exposures (Tables 20 and 21) and rates of abnormalities are similar to control rates.

A teratogenic response from the DBP exposures is evident in the rates of vertebral fusion and neural and hemal spine deformity (Tables 22 and 23). Histograms of such effects (Figs. 21 and 22) dramatically illustrate a dose-response relationship.

There are no clear dose-response relationships in the distribution of skeletal abnormalities in bromoform exposures (Tables 24 and 25). Except for dorsal fins, rates of abnormalities were similar to control rates. Abnormal dorsal fins were fairly common among offspring exposed to bromoform (Table 24); however, there were no abnormal dorsal fins in offspring reared in uncontaminated water (Table 25).

## DISCUSSION

The basic design of this study was aimed at distinguishing between teratogenic effects, that is, malformations resulting from abnormal embryological development, and chronic exposure effects, or abnormalities

Figure 19a-d. Photomicrographs of cleared and stained Rivulus marmoratus. a-Specimen (and parental adult) was continuously exposed to 0.740 mg/ℓ dibutyl phthalate (DBP) for entire life. Note extreme vertebral fusion, "kinks" and abnormal neural and hemal spines. b-Specimen reared in uncontaminated water from parental adult exposed to 0.370 mg/ℓ DBP. Note vertebral fusion in caudal region, abnormal neural and hemal spines and reduced pectoral fins. c-Specimen (and parental adult) was continuously exposed to 4.2 mg/ℓ bromoform for entire life. Note vertebral fusion, abnormal neural and hemal spines, abnormal dorsal fin pterygiophores and reduced pectoral fins. d-Specimen reared in uncontaminated water from parental adult exposed to 2.1 mg/ℓ bromoform. Skeleton normal. Scale applies to a,b,c and d.

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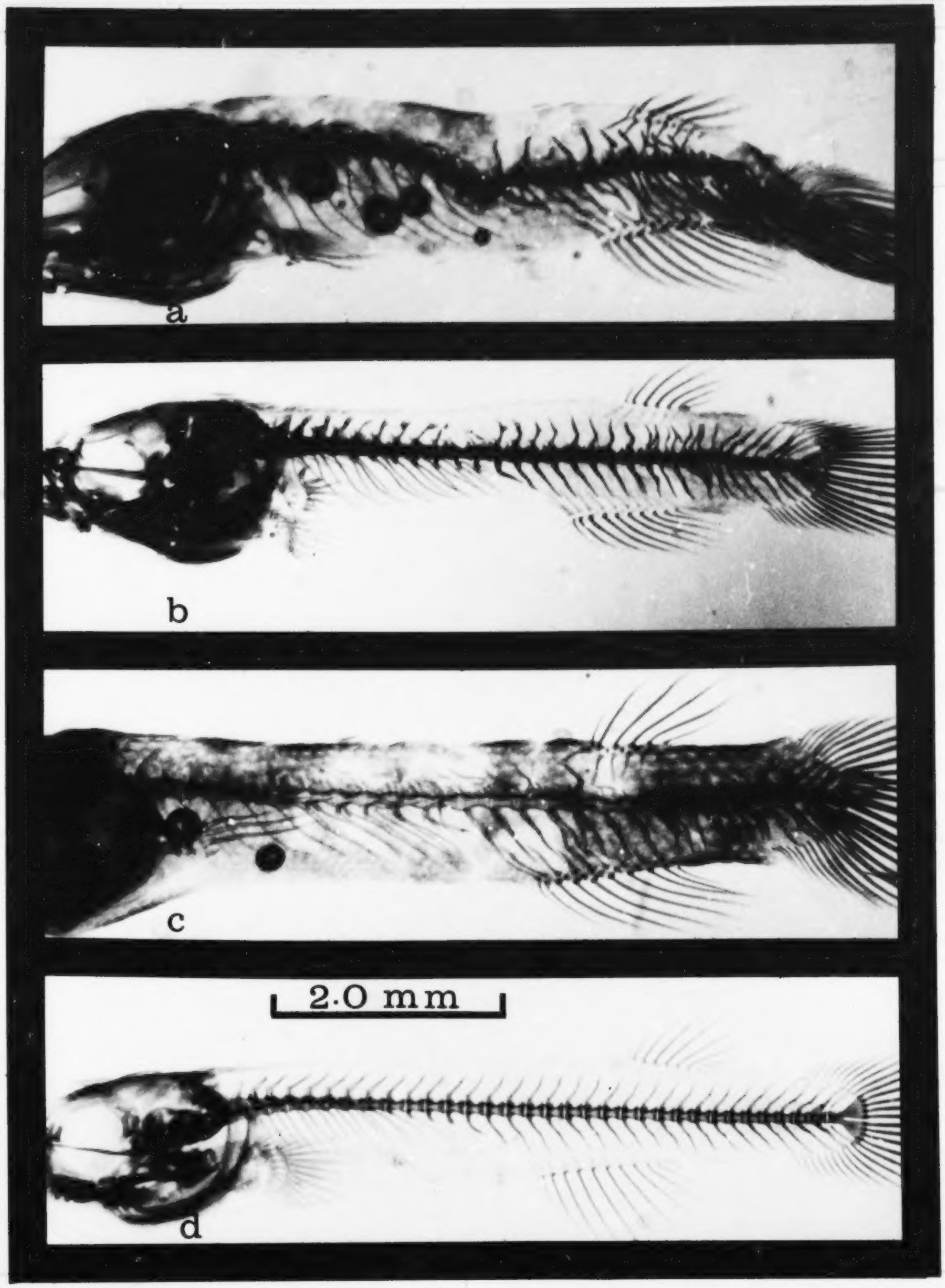

12/11
DBP-H
#113

12/13
DBP M CC
#54

12/22
BRO-H
#414

12/18
BRO-CC
#47

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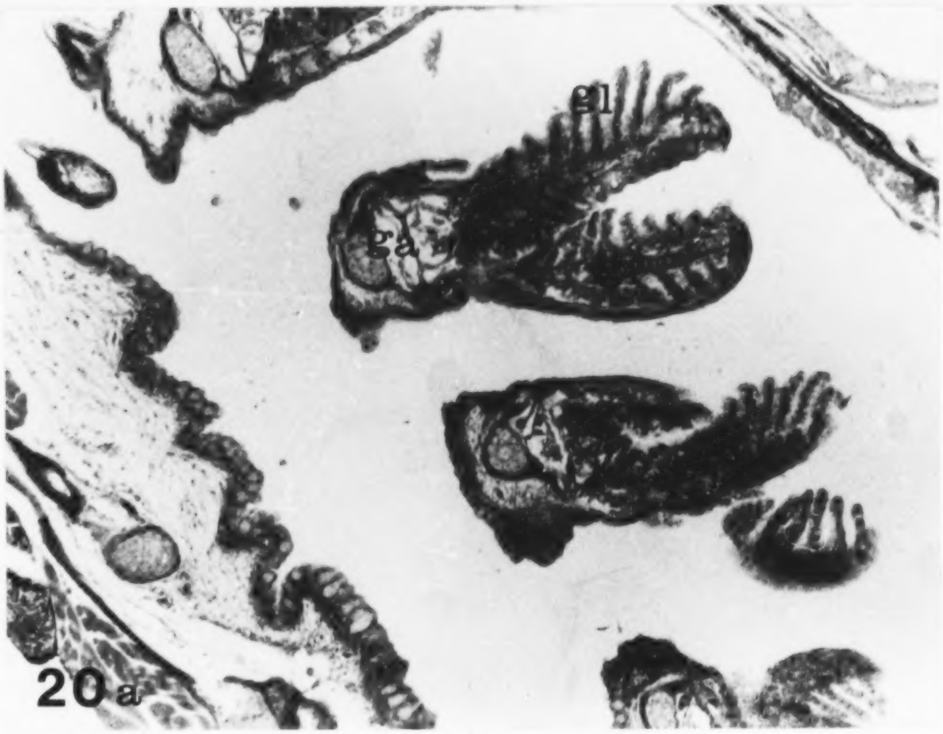
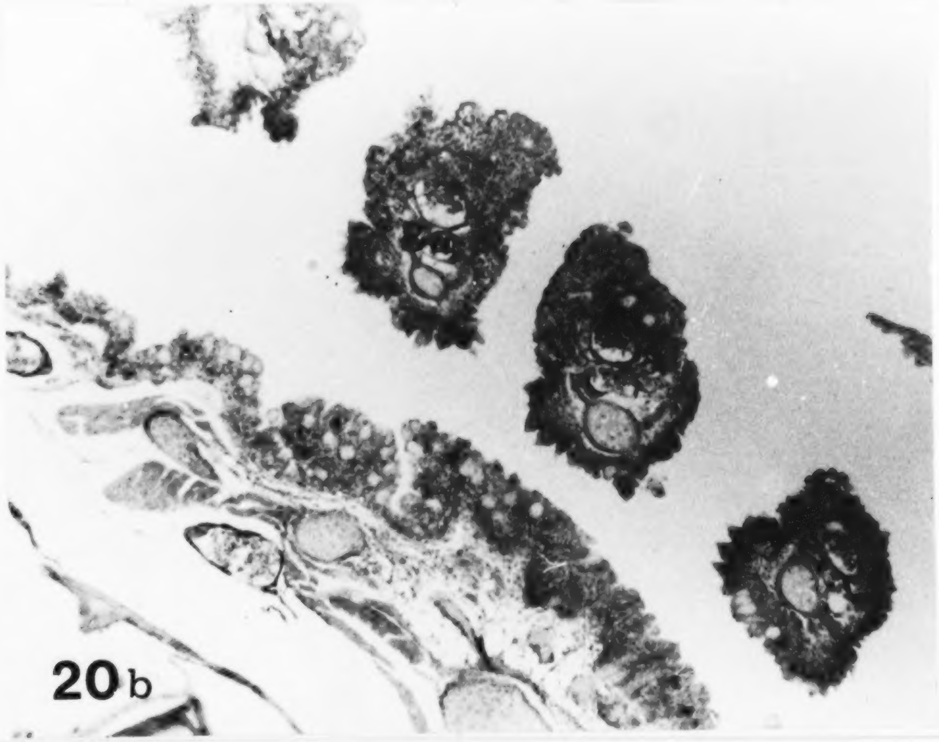
Figure 20. (a) Photomicrograph of a cross section through the gill arches (ga) of Rivulus marmoratus showing normal gill filaments (gf) and gill lamellae (gl), and (b) R. marmoratus exposed to 2,3,4,6-tetrachlorophenol (0.220 mg/ℓ) for entire life (4 months). Note complete absence of gill filaments and lamellae. x100.



20a



20b

Figure 21. Histogram of percent of various skeletal abnormalities in offspring of Rivulus marmoratus. Parental adults and offspring were exposed chronically to dibutyl phthalate (DBP) in their culture water in the following concentrations (mg/ℓ): 0.740(h), 0.370(m), 0.185(l). Parental adults and offspring of controls (c) were reared in uncontaminated water.

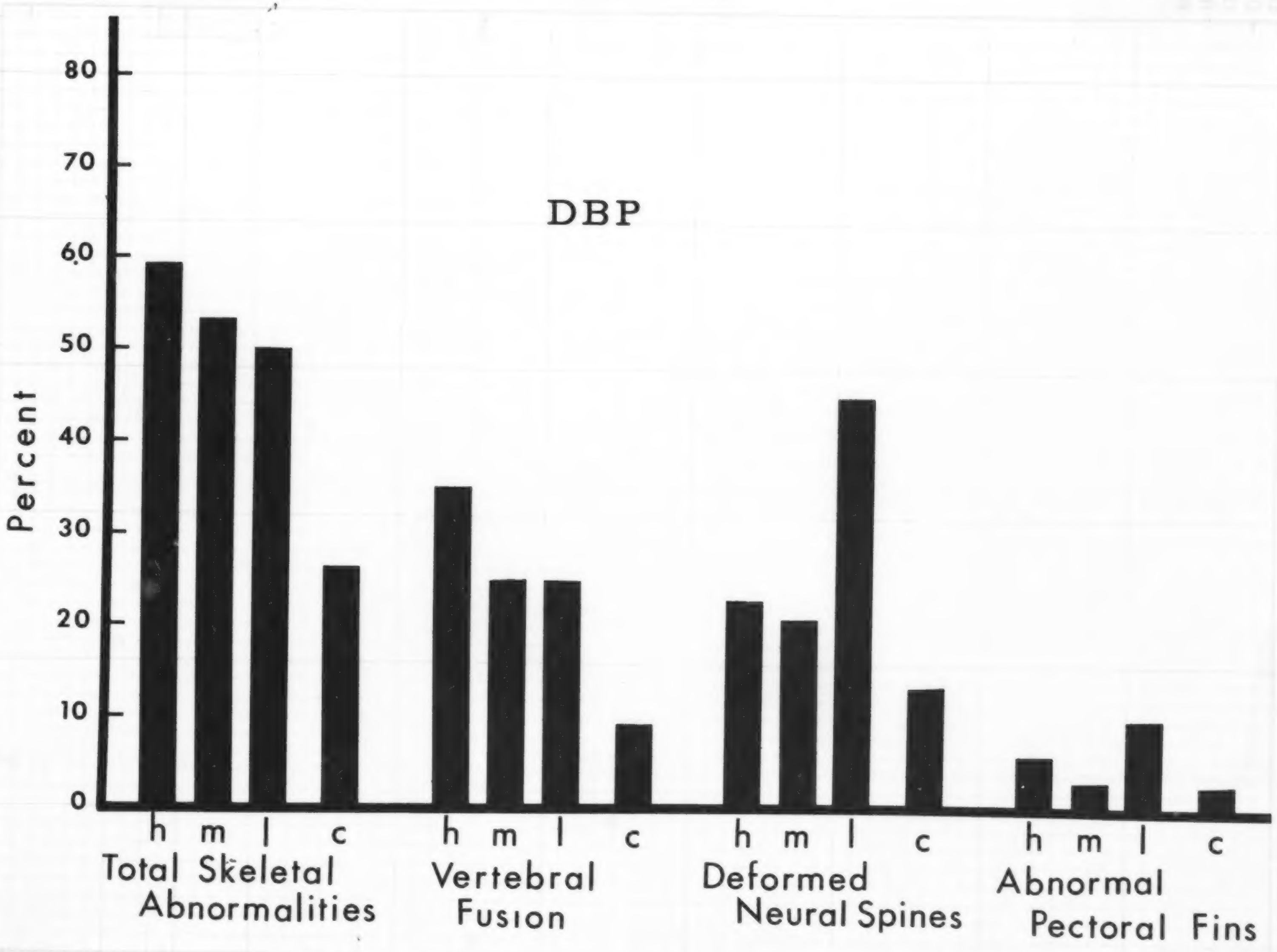
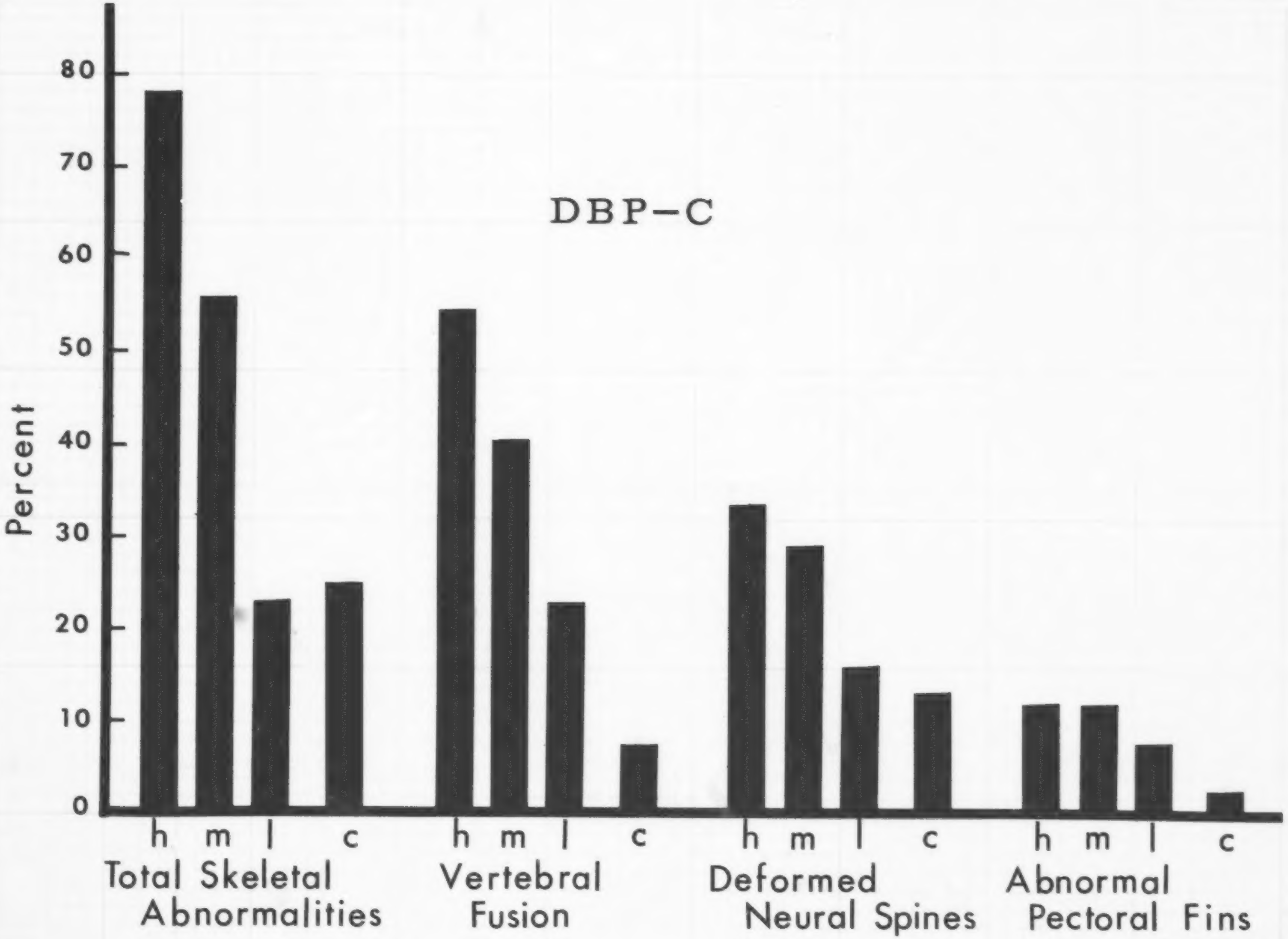

DBP
Percent
0
10
20
30
40
50
60
70
80
h
m
l
c
Total Skeletal Abnormalities
h
m
l
c
Vertebral Fusion
h
m
l
c
Deformed Neural Spines
h
m
l
c
Abnormal Pectoral Fins

Figure 22. Histogram of percent of various skeletal abnormalities in offspring of Rivulus marmoratus. Parental adults only were exposed chronically to dibutyl phthalate (DBP-C) in their culture water in the following concentrations (mg/ℓ): 0.740(h), 0.370(m), 0.185(l). Offspring of exposed parental adults and parental adults and offspring of controls (c) were all reared in uncontaminated water.


DBP-C
Percent
80
70
60
50
40
30
20
10
0
h m l c
Total Skeletal Abnormalities
h m l c
Vertebral Fusion
h m l c
Deformed Neural Spines
h m l c
Abnormal Pectoral Fins

caused by continuous exposure of post-embryonic developing offspring to chemical insult. Exposure concentrations were kept relatively low (20, 10, and 5% of the larval 96 hr LC50) and all phases of the life cycle were impacted to simulate contaminated conditions in natural waters. However, exposure levels were high enough to produce observable frequencies of effects using a limited number of animals.

During the course of the experiments actual chemical concentrations in the exposure containers were not determined. Solutions of test chemicals for the LC50 experiments were renewed daily; however, solutions for the chronic exposures were undoubtedly lower than nominal due to adsorption, volatilization, and/or breakdown of the test chemicals. For example, Laughlin et al. (1978) showed nearly complete loss of DBP from a seawater solution over a 24 hr period. Our exposures can therefore be considered more pulse-like than continuous. Future experiments of this nature might gain greater precision by exposure of test animals in a continuous-flow dilution apparatus.

The percent hatch in control fish was about 67%. This was the lowest hatching rate (except for the 4.2 mg/ℓ bromoform exposure) among all experiments. The highest consistent hatching rate was from those fish exposed to TRCP. The most plausible explanation for this pattern is that the chemicals had a stimulatory effect on hatching. Many cyprinodontid fishes delay hatching (see pp.20 and 21 Section 4 of this report) and rivulus is no exception. Often rivulus embryos would delay hatching to the point of exhausting yolk reserves and would die within the chorion. Harrington and Crossman (1976) routinely mechanically dechorionated stage 32 rivulus embryos to avoid this problem. Often embryos could be stimulated to hatch by changing the incubating medium from the synthetic seawater to natural seawater. Also, a weak ethanol solution stimulates hatching (W. P. Davis, personal communication). Rates of skeletal abnormalities and mortality among TRCP exposed fish and controls were comparable (Tables 10 and 13) but hatching rates differed greatly. At the levels used TRCP may have been an irritant to the point of stimulating hatching.

Mortality of offspring was high and somewhat variable in all exposures except TRCP where it was consistently lower than that of controls. In addition to a possible stimulatory effect on hatching, TRCP may have had a bacteriacidal effect which could have actually improved culture conditions.

The rate of skeletal abnormality in controls was 29.8% (Table 10). This compares well with the data of Lindsey and Harrington (1972) and Harrington and Crossman (1976). In their studies of temperature induced meristic variation in rivulus they reported rates of skeletal abnormalities between 25 and 38% in fish held constantly throughout life at 25-26$^{\circ}$C. In their study, rivulus held at 31$^{\circ}$C had skeletal abnormality rates between 87 and 100%. Also, low temperature (ca. 19$^{\circ}$C) induced skeletal abnormality rates between 53 and 88%. It is clear then that the spontaneous rate of skeletal abnormality in the three clones of rivulus held at standard culture conditions is around 30% and abnormally high and low temperatures induce skeletal abnormalities beyond this rate. It is difficult to compare types of abnormalities between this study and those cited because detailed descriptions of the abnormalities were not given. Preliminary results with wild-caught rivulus from near Naples, FL indicate that the spontaneous rate of skeletal abnormality is near zero; however, the response to temperature increase is similar to that of the fish derived from Harrington's clones (personal observations). Future studies of this nature which utilize wild-caught clones could be even more fruitful.

In some of the exposures it is difficult, because of low sample numbers, to discern an increased abnormality rate over controls. Because skeletal abnormalities were among several possible responses, too few animals were retained, in many cases, for clearing and staining procedures. Also, a number of fish were preserved in Bouin's fluid which decalcifies bone and precludes effective clearing and staining. However, overall the data demonstrated the sensitivity of rivulus to environmentally (chemically) induced skeletal malformations. For example, DBP has been shown to produce teratogenesis in rats when injected (ip) into pregnant females on the 5th, 10th, and 15th day of gestation at rates of 33%, 20%, and 10% of the LD50 (Singh et al., 1972). Rates of skeletal abnormalities in treated rats were 33.3%, 24.1% and 20.7%, respectively; control rats showed no abnormalities. In rivulus this known teratogen induces several types of skeletal abnormalities (Tables 22 and 23, Figs. 21 and 22). Considering its widespread occurrance in marine (Giam et al., 1978) and freshwater (Mayer et al., 1972) environments, these results could be significant, especially in view of the fact that DBP bioaccumulates in certain aquatic animals to as much as thousands of times the water concentration (Sanders et al.,1973).

Rates of skeletal abnormalities resulting from TRCP exposure (Tables 20 and 21) are approximately the same as control rates, indicating no teratogenic effect. Also, there were no overt chronic effects or pathologic changes resulting from continuous exposure to TRCP.

There are indications that TECP induces skeletal abnormalities, specifically vertebral fusion and abnormal neural and hemal spines (Tables 18 and 19). This is clearly a teratogenic response because elevated rates of abnormality appear in both exposed and unexposed offspring from exposed parental adults. However, erosion of fin rays and gills (Table 18, Figs. 16a-c and 20b) with associated epithelial hyperplasia occurred as the result of chronic exposure of the offspring to TECP. Fin erosion continued in some exposed fish until the complete fin structure was lost. Pectoral fins usually first showed signs of erosion, followed by median fins. TECP was clearly the primary causative agent; however, it is not known whether secondary causative agents (bacterial, fungal or viral) were also involved. Gill erosion in its most severe form (Fig. 20b) involved nearly complete loss of gill filaments with loss of associated lamellae. These fish undoubtedly survived to this state of extreme loss of primary respiratory surface by utilizing cutaneous respiration. Under natural conditions, in situations of drought, rivulus is capable of surviving out of water under moist leaf litter (personal observations). In this state of semi-dormancy the fish maintain a reduced metabolic rate and apparently utilize the skin as the major site of respiratory gas exchange (Abel, 1981).

Fin erosion has been observed in natural populations of other species of marine fish in several coastal locations: in the New York Bight (Mahoney et al., 1973), off southern California (Mearns and Sherwood, 1974) and in the estuary of the Duwamish River, Seattle, Washington (Wellings et al., 1976). In each case the highest incidence of fin erosion was observed in areas heavily contaminated with industrial and domestic pollution. Although the investigators were unable to determine the primary causative agent, a high correlation between the occurrence of the disease and the presence of various pollutants (heavy metals and halogenated organics) led them to suggest these as probable eteological factors. TECP induced fin erosion in rivulus strongly corroborates the contention that certain halogenated organics play a dominant role in the cause of the disease. The mechanism of TECP induced fin and gill erosion warrants further investigation.

An unusual result in this study was the development of abnormal dorsal fins in rivulus exposed chronically to bromoform (Table 24 and Figs. 17a-c). No dorsal fin abnormalities were noted in offspring of bromoform exposed adults which were transferred to uncontaminated water (Table 25). This indicates that bromoform exerts its effect during later development. The stage of development at which the environmental insult takes place determines, in large part, the resulting abnormality (Stockard, 1921). Embryos from bromoform exposed adults that were transferred to uncontaminated water (Table 25) may have been transferred before the stage at which dorsal fin development took place (stage 32; see Section 2 of this report). Because bromoform depurates from organisms rapidly (Gibson et al., 1980) it probably had little or no effect on development after eggs were removed from the contaminated solutions. In future experiments of this type embryos should be transferred to the respective conditions at a fixed stage, probably the terminal stage of embryonic development, to avoid ambiguity.

There have been a number of studies on fish teratogenesis (for review see Laale and Lerner, 1981). Most have involved direct exposure of eggs to chemical or physical perterbations at specific stages or during the entire period of embryological development. Although many such studies are involved with the elucidation of the mechanisms of teratogenesis in fish, some purport to ascertain environmental relevance and therefore involve the exposure of embryos of various species to known pollutants. In many of these studies egg exposure levels are chosen at or near the lethal level of the embryo without regard for other life stage susceptabilities. In developing a realistic evaluation of the potential of a chemical to induce congenital malformations or pathologic changes due to chronic exposure, the physico-chemical properties of the pollutant and levels of exposure are most important considerations. In nature, all stages of the life cycle would likely be exposed to the pollutants in question in varying degrees, depending upon the ecology of the species. Many studies involving direct exposure of eggs ignore the importance of the natural environment. Organic pollutants with high partition coefficients tend to bioaccumulate (Kopperman et al., 1979) and may be transferred (unchanged or after metabolic alteration) directly to the developing oocytes. There the xenobiotic may become incorporated into specific portions of the yolk and its ultimate effect on the developing embryo will depend upon the time and extent to which it is mobilized from the yolk reserves. For example, DDT and mirex, two highly lipophilic, persistent pesticides have been shown to accumulate in the eggs of the diamond killifish, *Adinia xenica*, when incorporated into the maternal test diet (Koenig, 1975). Negligible effects were observed during embryological development; however, dose-related mortalities occurred soon after hatching. The vast majority of these two pesticides were apparently confined to the triglyceride fraction of the yolk which was metabolized by the larvae soon after hatching. In cases such as these the embryo is not the target. Laboratory studies should be designed with the natural route of exposure in mind.

It is also possible that various pollutants may alter the maternal physiological state or the biochemistry of egg maturation which, in turn, could result in abnormal or less fit offspring. An analogous situation in certain birds is the well-known example of egg shell thinning resulting from altered calcium metabolism due to DDE exposure.

It is thus apparent that before a meaningful assessment of the teratological potential of a particular pollutant can be made, the maternal component and its indirect effects on early ontogeny must be included. Direct effects of a potential teratogen should be evaluated only at dosage levels at or lower than those tolerated by other life stages, otherwise data will lose environmental relevance.

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# SECTION 8

## MUTAGENESIS BIOASSAY: INVESTIGATIONS WITH *RIVULUS MARMORATUS* AND OTHER SELECTED FISH SPECIES

### INTRODUCTION

The aims of the investigations reported in this section were twofold: 1) to characterize the genetic system of *Rivulus marmoratus* and to evaluate the consequences of this genetic system for long-term, whole body exposure bioassays for the mutagenic effects of chemicals, and 2) to investigate possible short-term procedures for assessing mutagenic effects of chemicals in the *Rivulus marmoratus* system. These aims were subsequently modified to include alternate species when we were unable to identify genetic markers in laboratory clones and wild populations of *Rivulus marmoratus* and when the karyotype of *R. marmoratus* proved to be unsuitable for cytogenetic analysis.

### Investigations with *Rivulus marmoratus*

The genetic system represented by *Rivulus marmoratus* is unique among vertebrate species. Individuals are predominantly self-fertilizing hermaphrodites (Harrington, 1961) although some individuals occur as primary or secondary males (Harrington, 1967). The individuals available in this laboratory (clones DS, NA, and M) were descended through at least 30 uniparental generations from individuals collected from the wild by Harrington (1963) near Vero Beach (DS and NA) and near Miami (M). Wild-caught individuals used in this study were collected near Marco Island, Florida in October, 1978. Laboratory clonal individuals are presumably self-fertilizing hermaphrodites which are homozygous for most genes (Harrington and Kallman, 1968) and are isogenic within but not between clones (Kallman and Harrington, 1964). These conclusions are based upon histocompatibility criteria applied to parents and offspring of interclonal crosses. Natural and artificial cross fertilization resulted in amphimixis and suggest that subsequent segregation in hybrid animals is random with respect to paternally- and maternally-derived chromosomes (Harrington and Kallman, 1968).

The purpose of the present investigation was to identify gene markers by starch gel electrophoresis to assess the genetic distance between clones and to verify the occurrance of self-fertilization and segregation of maternally- and paternally-derived alleles during subesequent generations of uniparental reproduction. A secondary objective was to utilize genetic markers to assess the relative importance of self and cross-fertilization and homozygosity and heterozygosity in natural populations. Our approach was

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to screen 1-3 fish of each clone and at least 20 wild fish for 14 enzyme systems representing an estimated 28 loci.

The fish utilized in this study were adult and the largest available at the time of this study. Laboratory clones had been maintained for 20 years by uniparental reproduction and isolated from other individuals as eggs. Wild *Rivulus marmoratus* were captured by dip net from an impounded area near Marco Island on the West Coast of Florida. Fish were captured over a two day period within 500 yards of each other. All fish were maintained in isolation and fed frozen brine shrimp.

Individual fish were sacrificed by cooling and skeletal muscle, liver and nervous tissue (eye and brain) were separated and homogenized in 2 volumes of distilled water. Homogenates were centrifuged and supernates were frozen at $-20^{\circ}$C in capillary tubes until they were used. Electrophoretic separations were performed on a horizontal starch gel system. Four buffer systems (Shaw and Prasad, 1970) were utilized as follows: lithium hydroxide, pH = 8.2 (LiOH), at 350V for 3.5 hours; tris-versene-boric acid, pH = 8.0 (TVB), at 200V for 3.0 hours; tris-maleic acid - EDTA, pH = 7.4 (TME) at 100V for 5 hours and tris-citrate, pH = 6.7 (TC6.7) at 180V for 3.0 hours. Gels were sliced horizontally and stained for the following enzymes (Shaw and Prasad, 1970): adenylate kinase (AK), acid phosphatase (AP), esterase (EST), glutamate dehydrogenase (GDH), alpha-glycerophosphate dehydrogenase (αGPDH), glutamate-oxaloacetate transaminase (GOT), glucose-6-phosphate dehydrogenase (G6PDH), isocitrate dehydrogenase (IDH), lactate dehydrogenase (LDH), peptidase (PEP), 6-phosphogluconate dehydrogenase (6PGd), glucose phosphate isomerase (GPI), and phosphoglucomutase (PGM). Indolephenole oxidase was scored on gels stained for dehydrogenases.

Skeletal muscle, liver and neural homogenates were run for 3-7 individuals on one gel. Samples for laboratory clones were run simultaneously for all three clones. One sample per tissue of a previously run laboratory clonal fish was included with samples from wild-caught individuals for at least one run for each enzyme. Isozyme patterns obtained for a particular tissue were judged identical for all fish. The absence of genetic differences among the individuals screened presented difficulty in the identification of isozyme bands with specific gene loci. Consequently the number of loci that were screened was estimated on the assumption that all fish were homozygous and each band thus represented a product of a separate gene locus. This tends toward an overestimation of the number of loci as some bands may represent heterodimers or heterotetramers of products from different loci (Table 26).

The lack of detectable electrophoretic differences between individuals separated by 20 years and hundreds of miles supports the view that the North American populations of *Rivulus marmoratus* were recently derived from a small founder population and are now expanding at a rapid rate (Fowler, 1928; Rivas, 1945; Harrington and Rivas, 1958; Tabb and Manning, 1961). The surprising absence of genetic variation between laboratory clones and within natural populations has resulted in the postponement of further analyses of the *Rivulus marmoratus* genetic system until suitable genetic markers can be identified perhaps from individuals collected in other parts

Table 26. Screening of Clonal and Wild-Caught *Rivulus marmoratus* for Biochemical Variation

| Enzyme[a] | Buffer[a] | Tissues[b] Examined | Maximum Number of Isozymes | Estimated Number of Loci/Fish | # of Animals Screened | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Clones | | | Wild-Caught |
| | | | | | DS | NA | M10 | |
| AK | TME | M, L, N | 1 | 1 | 2 | 2 | 2 | 25 |
| AP | TME | M, L, N | 1 | 1 | 2 | 2 | 2 | 18 |
| EST | LiOH | M | 4 | | | | | |
| | | L | 2 | | | | | |
| | | N | 1 | 4 | 3 | 2 | 3 | 28 |
| GDH | TME | M | 3 | | | | | |
| | | L, N | 2 | 3 | 1 | 1 | 1 | 30 |
| GPDH | TVB | M | 1 | 1 | 2 | 2 | 2 | 29 |
| GOT | TVB | M, L, N | 2 | 2 | 1 | 1 | 1 | 32 |
| G6PDH | TVB | M, L | 1 | | | | | |
| | | N | 2 | 2 | 2 | 2 | 2 | 21 |
| IPO | TVB | M, L, N | 1 | 1 | 2 | 2 | 2 | 18 |
| IDH | TC6.7 | M, L, N | 2 | 2 | 0 | 0 | 0 | 36 |
| LDH | TVB | M | 2 | | | | | |
| | | L | 1 | | | | | |
| | | N | 3 | 3 | 3 | 3 | 3 | 29 |
| PEP | LiOH | M, L, N | 2 | 2 | 2 | 2 | 2 | 28 |
| 6PGD | TC6.7 | M, L, N | 1 | 1 | 2 | 2 | 2 | 15 |
| GPI | LiOH | M, L, N | 2(+) | 2(+) | 0 | 0 | 0 | 25 |
| PGM | TC6.7 | M, N | 3 | | | | | |
| | | L | 1 | 3 | 2 | 2 | 2 | 17 |

[a] See methods section for abbreviations of enzymes and buffer systems.

[b] Abbreviations for tissues: M=skeletal muscle; L=liver, N=brain and eyes

of the range of this species. The absence of electrophoretic variation across 20-28 loci is surprising in view of the histocompatibility differences reported by Harrington and Kallman (1968). Perhaps the use of histocompatibility criteria screens for a very much larger number of possible loci (subject to mutation) than are screened for by electrophoretic techniques.

## *Rivulus marmoratus*: Short Term Assays

The utilization of short term *in vitro* procedures to assess the effects of potentially mutagenic and carcinogenic chemicals and the investigation of mechanisms by which these chemicals exert their toxic effects has progressed rapidly in the past 10 years. The ability to employ *in vitro* procedures to investigate the effects or lack of effects of specific toxic chemicals in whole animal bioassays is important in the overall evaluation of a test organism. To this end we have attempted to establish a permanent line of cultured cells derived from *Rivulus marmoratus* to provide sufficient quantities of material to investigate the metabolism of mutagens and carcinogens and to evaluate sensitivity to induction of point mutations, chromosomal mutations, DNA repair and neoplastic transformation by these chemicals. An evaluation of the *Rivulus marmoratus* karyotype for cytogenetic analyses was also conducted. Procedures and results are discussed in the following section.

## Investigations of Alternate Species

Recent progress in the use of cellular and subcellular effects to assess the toxicity of chemicals in a variety of genetic systems from bacteria to man have greatly extended our ability to screen large numbers of chemicals for potential sublethal mutagenic and carcinogenic activity. Concurrent development of similar procedures for teleost fish systems might be expected to provide aquatic toxicologists with useful assays for the detection, identification, and assessment of the effects of waterborne mutagenic and carcinogenic hazards in aquatic ecosystems. The development of short term assay procedures for a suitable teleost species could also be expected to enhance the ability of aquatic toxicologists using whole animal exposure assays to investigate at the cellular and subcellular levels the causes of differences between teleost fish systems and mammalian systems. Potential sources of interspecific differences in response to exposure to genotoxic chemicals include differences in metabolism, pharmacokinetics, DNA repair and epigenetic phenomena associated with neoplastic transformation. The purpose of this series of investigations were: 1) to survey a number of teleost species, including *Rivulus marmoratus*, as possible test organisms for the development of short-term procedures to assess the effects of genotoxic chemicals in laboratory and field situations, 2) to evaluate the feasibility of these procedures for use by aquatic toxicologists, and 3) to develop *in vitro* procedures for investigating metabolism of xenobiotics, DNA repair and neoplastic transformation in fish cells.

Among the short term assays that have attained credibility as indicators of the genetic activity of chemicals, sister chromatid exchange (SCE) analysis is possibly the most sensitive and versatile (Perry and Evans, 1975;

Perry, 1980). SCE analysis is a cytogenetic assay that is more sensitive than conventional analysis of chromosomal aberrations for assessing the genotoxic activity of chemicals and SCE analysis can be adapted to a variety of *in vitro* test protocols that are essential for applications in aquatic toxicology. The primary criterion that we have applied in selecting a suitable species for further development was the suitability of the karyotype for cytogenetic analysis. A second feature considered desirable for a test organism for sublethal effects is a tolerance for extensive manipulation in the laboratory and for adverse conditions that are expected to be associated with exposures to waterborne chemicals. A fourth consideration is that a test organism be readily available to laboratories which would potentially have use for the assays to be developed. A final consideration for a test organism to be used in field studies is preference for association with bottom sediments where many chemicals tend to accumulate.

## MATERIALS AND METHODS

### Collection of Fish

The *Rivulus marmoratus* used for karyotype examination and for tissue culture were obtained from laboratory stocks maintained as described in an earlier section. Other species that were examined were obtained from freshwater and estuarine systems in the Charleston area. The freshwater species *Umbra pygmaea* (DeKay), *Fundulus chrysotus* (Gunther) and *Chologaster cornuta* (Agassiz) were collected with a dip net. The estuarine species *Symphurus plagiusa* (Linnaeus), *Paralichthyes dentatus* (Linnaeus) and *Citharichthyes spilopterus* (Gunter) were collected in trawls. *Opsanus tau* (Linnaeus) were collected in crab traps.

### Cytogenetic Methods

Numerous procedures have been described for the preparation of metaphase chromosomes for cytogenetic analysis in fish. The technique with greatest potential for application to mutagenesis studies, including SCE analysis, is that of Kligerman *et al.*, (1975), Kligerman and Bloom (1976) and Kligerman (1979) and was used for the initial cytogenetic screening of potential test organisms. Fish were injected ip with 25 ug of colchicine per gram body weight and held in well-aereated aquaria for 6 hours before sacrificing. Gills, stomach and intestine were excised, cut into short segments, treated with hypotonic KCl,fixed with three changes of methanol-acetic acid (3:1) fixative and stored at 5°C until used. Slides were prepared by mincing tissue pieces in 50% acetic acid until a slurry of cells was visible (about 1 minute). The cell suspension was drawn into a 100 μℓ pipet, expelled onto a microscope slide at 50°C and withdrawn from the slide to leave a ring of cells 1 cm in diameter. Slides were air dried and stained for 8 minutes in 4% Giemsa in distilled water.

The most effective technique for obtaining abundant, well spread, and clearly defined metaphase chromosomes in many mammalian systems has been the culture of peripheral leukocytes in the presence of a mitogen. Few reports, however, have demonstrated the successful extension of this procedure to teleost fish systems (Gold, 1979). We were able to culture leukocytes from the oyster toadfish. Subsequent cytogenetic analyses in this species were done with peripheral leukocyte cultures (Maddock and Kelly, 1980). Blood samples were obtained by cardiac puncture using a heparinized syringe. Whole blood (0.5-1.0 ml) was expressed into 5 ml of RMPI 1640 (GIBCO) medium supplemented with 15% fetal calf serum, antibiotics, sodium chloride and phytohemagglutinin (GIBCO). The pH of cultures was adjusted by gassing tubes with 5% $CO_2$ in air. At harvest 6-8 days later cultures were treated with clolcemid for 5 hours, centrifuged, resuspended in hypotonic KCL for 10-15 minutes and fixed with three changes of methanol-acetic acid (3:1). Cells suspended in fixative were dropped onto glass slides and air dried. For conventional staining, slides were placed in 4% Giemsa for 8 minutes.

To obtain sister chromatid differentiation 5-bromo-2'-deoxyuridine (Sigma) was added to culture medium before seeding with blood. Differential staining was accomplished by staining in the fluorescent dye Hoechst 33258, exposure to a 40W blacklight fluorescent bulb and restaining for 10 min. in 2% Giemsa in phosphate buffer (pH = 6.8) after the method of Goto *et al.* (1975). *In vitro* exposures of dividing leukocytes to the test chemicals ethyl methanesulfonate, EMS (Sigma) and bromoform, BF (Fisher) were conducted by additions within 24 hours of culture initiation for the duration of the culture period. All cultures were maintained at 26°C.

### Assays for *Samonella* Mutagenesis

Preparation of S-9 and plate assays for *Salmonella* mutagenesis were conducted with minor modification of the methods described by Ames *et al.* (1975). The concentration of S-9 protein per gram of toadfish hepatic tissue is lower than that of rat hepatic S-9. Consequently liver tissue from toadfish was homogenized in KCl at a 1:2 dilution and final protein concentrations of rat and toadfish liver S-9 were prepared on a protein for protein basis (Lowry *et al.*, 1951). Plate assays were incubated for 48 hrs at 37°C when rat S-9 was used and for 12 hrs at 26°C then for 48 hrs at 37°C when fish S-9 was used. Animals (rats and fish from 200-300 g) from which S-9 was to be prepared received either no pretreatment or were injected IP with 200 mg/gram body weight of Aroclor (AC) in corn oil five days prior to sacrifice or with 0.02 mg/g body weight of 3-methylcholanthrene (MC) 5, 3, and 1 days before sacrifice. Liver S-9 fractions were frozen at -80°C until use. The precarcinogens utilized in these assays, benzo(a) pyrene (BP), and 2-aminoanthracene (AA) (Aldrich) were dissolved in DMSO and stored at 5°C in DMSO between experiments.

### Tissue Culture

In initial attempts to initiate primary cell cultures from tissues of *Rivulus marmoratus* and *Opsanus tau* we have used two methods: direct planting (explant) and trypsin dispersion (Wolf, 1973). Tissues to be cultured by direct planting methods were excised into sterile Hank's BBS and minced with scissors for 7-10 minutes. The resulting suspension of small tissue pieces was added (4-6 drops) to 4 ml of growth medium in a culture flask with 25 cm$^2$ surface area. Medium and unattached tissue pieces were drained and replaced by fresh growth medium after 1-1.5 hours. Medium was changed at weekly intervals until a monolayer of cells was obtained. Tissues to be dispersed by trypsin were minced and washed with Hank's BSS and tissue pieces placed in a trypsinization flask in a refrigerator at $5^{\circ}$C and stirred with a magnetic stirrer for 30-90 minutes in ATV dispersion mixture (Nadin and Darby, 1958). The resulting fluid and dispersed cells were discarded and remaining tissue pieces were resuspended in ATV and stirred several hours to overnight at $5^{\circ}$C. The resultant suspension of cells was centrifuged, the supernate discarded and the pelleted cells washed and resuspended in growth medium to give 1-3 x $10^5$ cells per ml. Culture flasks were seeded with 4-5 ml of cell suspension. Medium was changed weekly until cultures showing new growth reached monolayer. Monolayer cultures from either of the procedures outlines above were subcultured by discarding old medium, rinsing with Hank's BSS, treating with three changes of ATV for 15 minutes, 15 minutes, and 30 minutes. Harvested cells were washed and then resuspended in growth medium and used to seed 2 new culture flasks. The growth medium used in all experiments was L-15 medium (GIBCO) supplemented with 15% fetal calf serum, antibiotics, MEM amino acids and MEM vitamins. Additional NaCl was added to increase the osmotic pressure of the medium. Cultures were maintained in sealed flasks at $26^{\circ}$C.

## RESULTS

### Cytogenetic Survey

There are a number of teleost species available in the Charleston area that from previously published reports could be expected to possess karyotypes that were suitable for detecting chromosome damage. The freshwater species *Umbra pygmaea* was closely related to the species *Umbra limi* that had been used by Kligerman *et al*. (1975) and at the time *U. pygmaea* had not been examined cytogenetically. The karyotype of *U. pygmaea* is similar to that of *U. limi* (Figure 23a) and is excellent for mutagenesis studies. *U. pygmaea* has subsequently been used to detect mutagenic activity in Rhine River water by SCE analysis (Alink *et al*., 1980). The freshwater species *Fundulus chrysotus* had been previously examined (Chen, 1971) and possesses a karyotype suitable for cytogenetic analysis (Figure 23b). Further studies of the two species were postponed because of their limited availability in the area and our greater interest in identifying a marine species for further study. Another freshwater fish examined was *Chologaster cornuta* (Figure 23C).

The primitive teleost karyotype is comprised of 24 pairs of small chromosomes (Denton, 1973). Many species of marine teleosts, including *Rivulus marmoratus* (Figure 24b) possess this cytogenetic structure and are not suitable for use as cytogenetic models in mutagenesis studies. Our approach was to examine local representatives of families that contain demersal marine species. The flatfish (Pleuronectiformes) and the toadfish (Batrachoidiformes) were surveyed. The blackcheek tonguefish (Cynoglossidae: *Symphurus plagiusa*) karyotype consists of 46 chromosomes that are quite small (Figure 23d). The summer flounder (Bothidae: *Paralichthys dentatus*) karyotype was comprised of 46 small chromosomes (Figure 24a). The oyster

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Figure 23. Metaphase chromosomes of various fish species prepared by the solid tissue technique (Kligerman and Bloom, 1977):

a. *Umbra pygmaea* (DeKay)
b. *Fundulus chrysotus* (Gunther)
c. *Chologaster cornuta* (Agassiz)
d. *Symphurus plagiusa* (Linnaeus)

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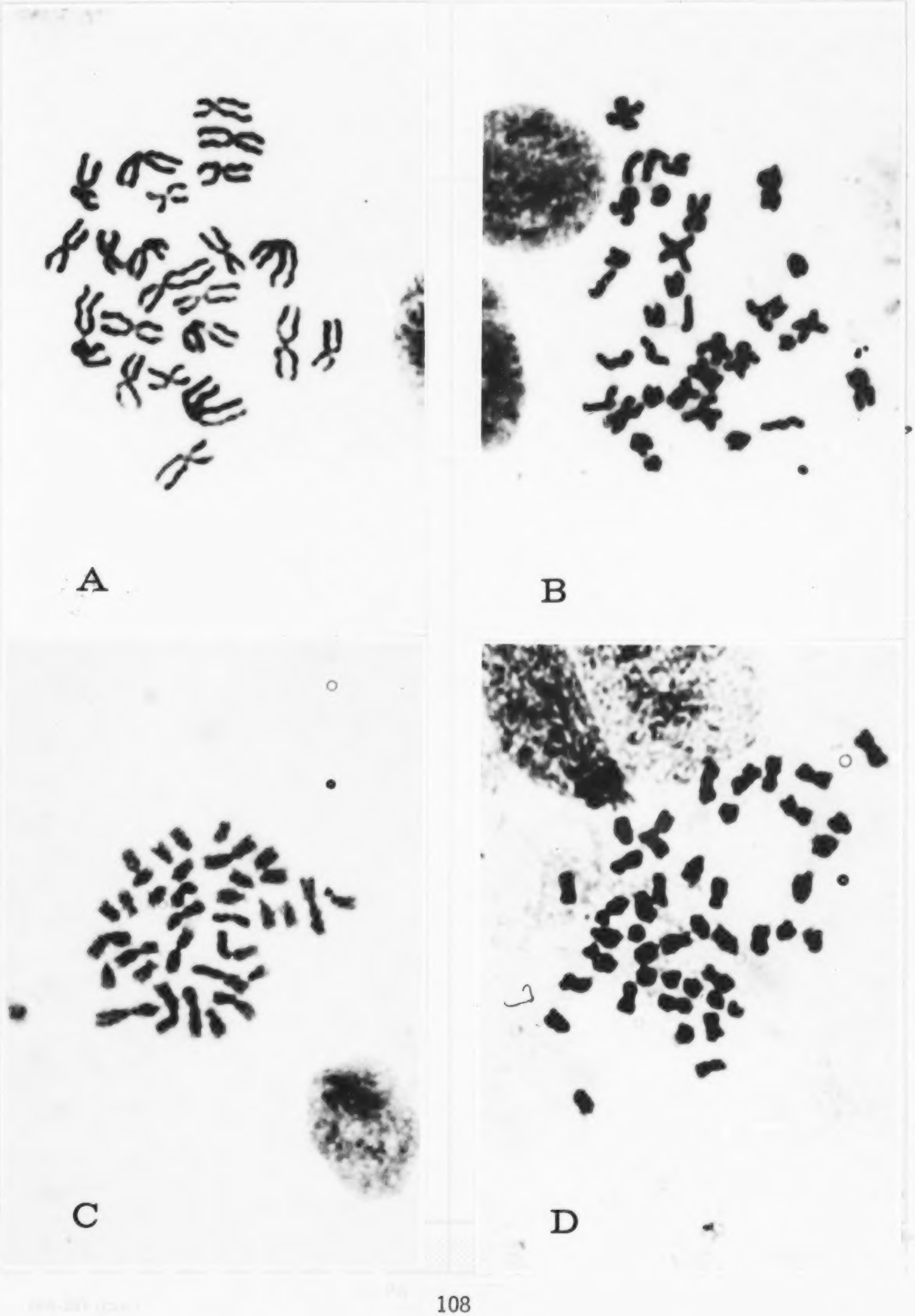
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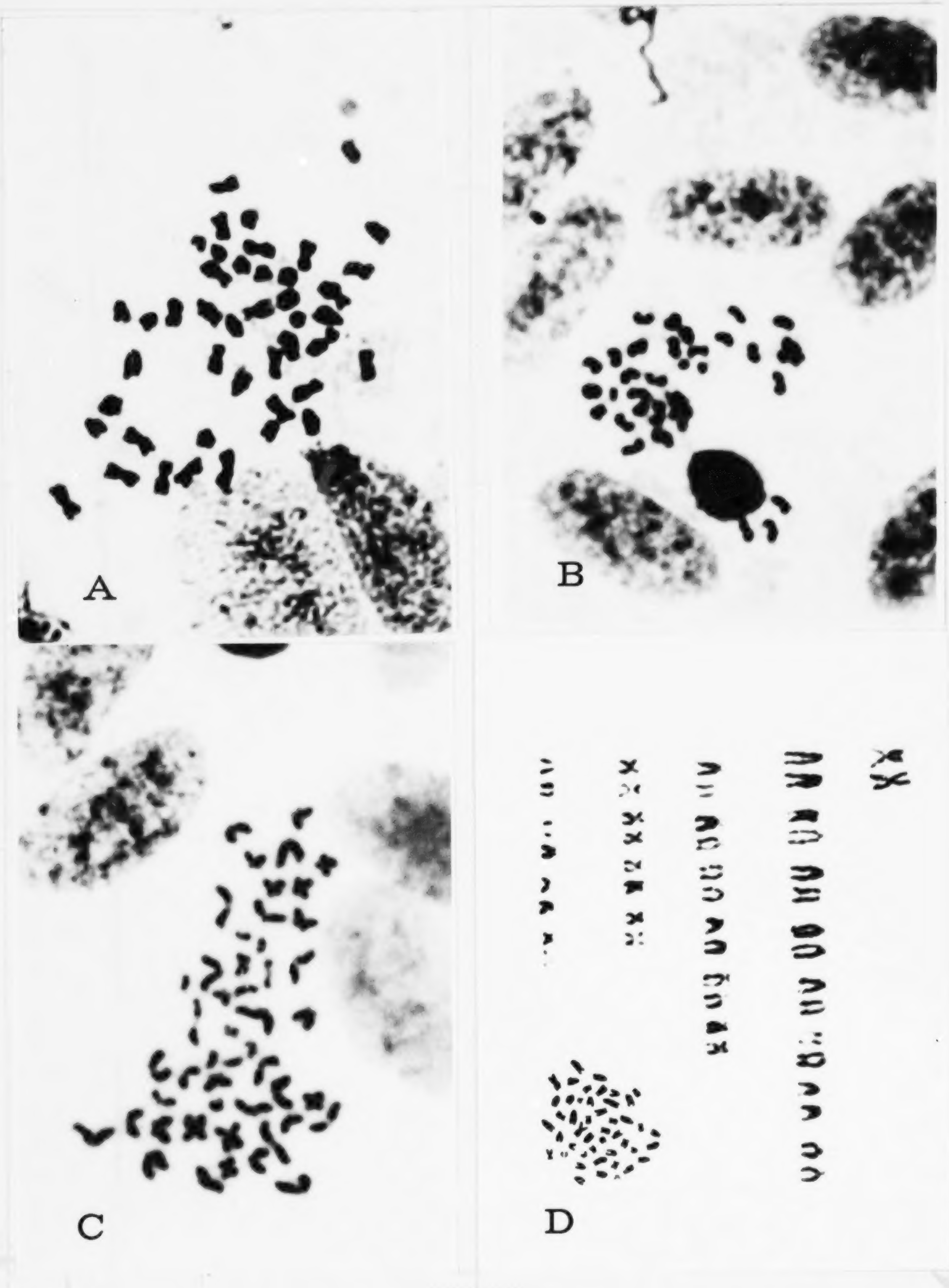
Figure 24. Metaphase chromosomes of various fish species prepared by the solid tissue technique (a-c) and by air drying of cultured leukocytes (d):

a. *Paralichthys dentatus* (Linnaeus)
b. *Rivulus marmoratus* (Poey)
c. *Opsanus tau* (Linnaeus)
d. *Opsanus tau* (Linnaeus)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toadfish (Batrachoididae; Opsanus tau) karyotype was comprised of 46 chromosomes, many quite large, and was quite suitable for cytogenetic analysis (Figure 24c). The promising features of the toadfish karyotype and its potential as a test organism by all criteria that were discussed earlier led to the discontinuation of the cytogenetic survey and increased efforts to evaluate the toadfish as a cytogenetic model.

Culture of peripheral leukocytes is one of the most effective methods for preparing metaphase chromosomes for cytogenetic analysis in mammalian species. Metaphase chromosomes from leukocyte cultures are abundant, well spread and have a well defined morphology. Leukocyte preparations are applicable to the assessment of mutagenesis in protocols with in vivo and with in vitro exposure to chemicals with genotoxic activity. Our attempts to culture toadfish leukocytes were successful (Figure 24d) and leukocyte culture has been the basis for our subsequent investigations.

## Sister Chromatid Exchange (SCE) Analysis in Dividing Toadfish Leukocytes

Sister chromatid exchange assays involve the counting of reciprocal exchanges between the genetically identical chromatids of replicated chromosomes at metaphase. A dose-response relationship between the concentration of a variety of known mutagens and carcinogens and the number of sister chromatid exchanges was demonstrated by Perry and Evans (1975) using Chinese hamster ovary cells in vitro. A requisite procedure in sister chromatid assays is the incorporation of bromodeoxyuridine (BUdr), an analog of thymidine, into DNA; this is usually done for two rounds of replication to provide chemical differentiation of DNA and subsequent visualization of exchange between otherwise identical sister chromatids. Presumably, one double-stranded DNA molecule comprises one chromatid of a metaphase chromosome. After two rounds of replication in the presence of BUdr, every metaphase chromosome has one chromatid with one unsubstituted and one BUdr-substituted strand of DNA and another chromatid containing two BUdr-substituted strands of DNA. Subsequent staining with a fluorescent dye (usually Hoechst 33258) or with a fluorescent dye followed by exposure to light and staining in Giemsa (fluorescent plus Giemisa or FPG technique) results in cytologically detectable sister chromatid exchanges.

A representative toadfish karyotype (Figure 24d) contains 46 chromosomes, including one pair of large metacentrics, eight pairs of large acrocentrics, six pairs of subtelocentrics, four pairs of small metacentrics and four pairs of small acrocentrics. These chromosomes are larger than those of all other marine species we have examined and are suitable for SCE analysis (Figures 25 a & b). In addition, the ease with which toadfish leukocytes may be cultured, a quality shared by few other fish species (Gold, 1979), their tolerance to unfavorable conditions and to repeated blood collection, and their association with estuarine sediments, where many pollutants tend to accumulate, combine to recommend the toadfish as a useful organism to explore the possibility of an SCE assay system for both field and laboratory applications.

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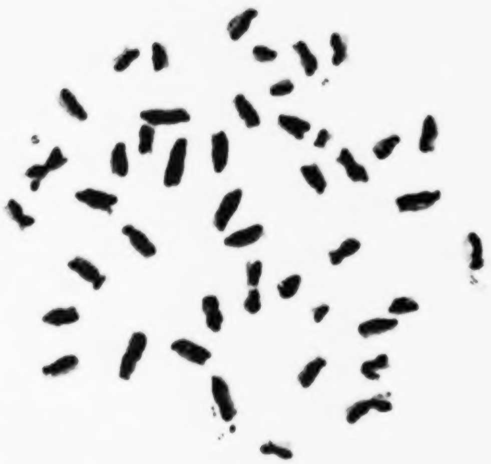
Figure 25. a. A second devision metaphase from a control culture of toadfish leukocytes showing sister chromatid differentiation of cell chromosomes and sister chromatid exchanges (10 exchanges were counted).

b. A second division metaphase from a toadfish leukocyte culture exposed *in vitro* to 62 μg/ml of EMS for 4 days prior to harvesting (22 exchanges were counted).

c. Monolayer of cells from a primary culture of *Opsanus* ovary tissue.

d. Monolayer of cells from a culture of *Opsanus* ovary tissue in the fourth passage.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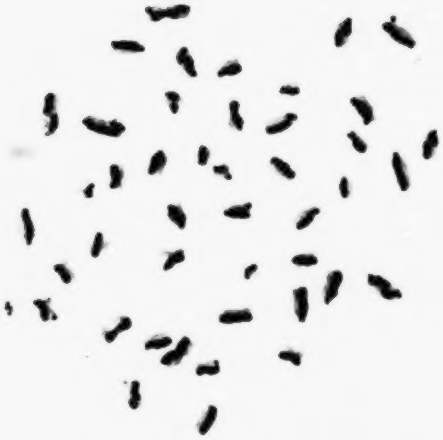
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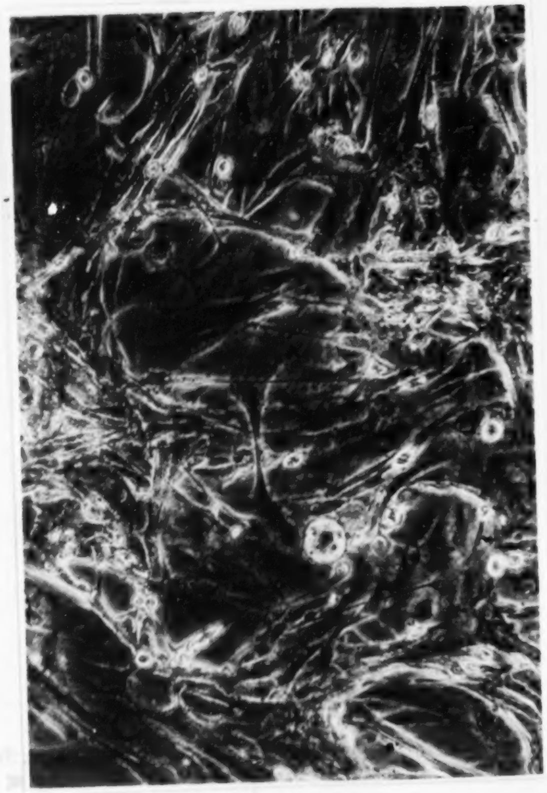

Exposure of dividing toadfish leukocytes to the mutagen-carcinogen EMS at dosages comparable to those reported for mammalian test systems indicate that SCE analysis of toadfish leukocytes is of comparable sensitivity (Table 27). Nine metaphases from two cultures exposed to 62 μg/ml of EMS yielded 192 exchanges for an average of 21.3 exchanges per metaphase. This is a significant increase over the control rate of 7.0 exchanges per metaphase obtained from 20 metaphases from two cultures. Exposure to 124 μg/ml of EMS yielded an average of 35.1 exchanges per metaphase, indicating a possible dose-dependent response of SCE rate to EMS exposure. Although these results are based on small samples of metaphases, they nevertheless demonstrate the feasibility of SCE analysis using a toadfish *in vitro* system.

Exposure of dividing leukocytes to bromoform at low concentrations did not result in increased rates of SCE (Table 27). Bromoform has been shown to be mutagenic in *Salmonella* and carcinogenic in mice. Perhaps the genetic activity of bromoform, if any, may prove to be detectable at higher concentrations or in tests *in vivo*, allowing for metabolic activation of compounds not mutagenic before activation. The volatility of bromoform and the necessity of periodic gassing of cultures with 5% $CO_2$ in air may have reduced the degree of exposures of leukocytes below the level of detection by SCE analysis. In addition, our lack of success in obtaining metaphases in glass culture tubes with Teflon-lined caps necessitated the exposure of cells to bromoform in polystyrene culture tubes (Falcon 3033). We are not aware of any previous reports of SCE analysis following exposure to bromoform in other SCE assay systems.

### Metabolism of Benzo(a) pyrene (BP) and 2-Aminoanthracene (AA) to *Salmonella* Mutagens by Toadfish S-9

The relative proportions of functionally distinct forms of cytochrome P450 enzymes which populate the endoplasmic reticulum of mammalian cells appear to be a major determinant of the degree to which promutagens and carcinogens are activated or detoxified. Although recent investigations indicate that metabolism of xenobiotics in fish is similar to that in mammals (Schoor and Couch, 1979; James and Bend, 1980) there are definite species differences even among rodents in the ability to activate specific mutagens and carcinogens. The nature of P450 systems of a teleost species proposed for use as a test organism for potential mutagens and carcinogens should be characterized. To this end we report a series of comparative studies to determine the effects of hepatic microsomal enzyme (S-9) preparations from rats and toadfish pretreated with standard enzyme inducers (3 methyl-cholanthrene (MC) and Aroclor (AC) and untreated on the metabolism of a polycyclic aromatic hydrocarbon and an aromatic amine to *Salmonella* mutagens.

Six concentrations of each test chemical (0.2-20.0 μg/plate) were tested in the presence of six different S-9 types (from untreated, MC-pretreated or AC-pretreated fish or rats). Each of the 432 data points were done in duplicate. Strain TA 98 was provided by Bruce Ames.

Table 27. Sister Chromatid Exchange (SCE) Rates Following In Vitro Exposure of Dividing Toadfish Leukocytes to Ethyl Methanesulphonate (EMS), Bromoform and Hank's (control)

| Chemical | Concentration (μg/ml) (molarity) | Number of Fish | Number of Cultures | No. SCEs / No. Metaphases | vs Control t-test |
|---|---|---|---|---|---|
| Control (Hank's) | | 2 | 2 | 139/20 | |
| EMS | 62(500 μM) | 1 | 2 | 192/9 | $p < 0.01$[a] |
| | 124(1000 μM) | 1 | 1 | 211/6 | $p < 0.01$[a] |
| BF | 0.10(0.40 μM) | 2 | 5 | 45/7 | $p > 0.05$ |
| | 100.00(400 μM) | 1 | 3 | 58/8 | $p > 0.05$ |

[a] Three metaphases from one control culture were scored for 24, 23, and 28 SCE. Because they represented an SCE rate at least twice as large as the largest from any other control culture, they were not included in this table. If they are included, the mean rate of SCE in control cultures becomes 9.3 SCE per metaphase, and the difference between SCE rate in control and EMS-treated cultures is not statistically significant, probably because of the small sample size.

The concentrations of S-9 protein from fish liver (90 mg protein/g wet liver) was lower than that from rat liver (160 mg protein/g wet liver) and, thus, the concentrations of S-9 per plate of fish and rat S-9 were adjusted to be equivalent on a mg protein/plate basis. Neither BP nor AA were mutagenic in absence of S-9 protein. The activation of AA was considerable for all fish and rat S-9's tested. All fish S-9 and S-9 from MC- and AC-pretreated rats activated BP but S-9 from untreated rats resulted in little, if any, activation of BP. The extent of activation of BP and AA under optimal conditions of S-9 type and concentration for fish (BP:937 revertants/plate and AA:6339 revertants/plate) and rat (BP:847 revertants/plate and AA:6692 revertants/plate) were comparable. The data summarized in Figure 26 show the effects of increasing concentrations of S-9 protein on a single concentration of test chemical. The curves are representative of results obtained for other concentrations of test chemical. It is apparent from the forms of the curves that pretreatment with MC but not with AC had a detectable effect on the activation of these test chemicals. As expected both MC and AC pretreatment had discernable effects on the metabolism of these test chemicals in the rat. These results support the growing evidence that the capacity of fish enzymes systems to metabolize promutagenic xenobiotics are similar in fish and mammals although the effects of inducers in these systems may not be similar. The lack of a detectable effect of phenobarbital pretreatment on xenobiotic metabolism in fish has been reported (Bend _et al._, 1973).

### Tissue Culture

The initiation of tissue cultures from small species is hampered by difficulty in obtaining gram quantities of any tissue needed for primary culture. Consequently, the results we have obtained in limited attempts to culture tissues from _Rivulus marmoratus_ have been discouraging (Table 28). On the other hand, the ready availability of tissues from toadfish has resulted in encouraging prospects for primary culture of toadfish tissues (Table 28). Primary cultures of toadfish liver, ovary, and skin have given monolayer cultures. Liver and ovary primary cultures have been successfully subcultured. We have had no success in culturing cells from the toadfish beyond the fourth passage. The toadfish cultures are of mixed cell type in the primary culture (Figure 25c) but appear to be predominantly epithelioid in later passages (Figure 25d). These results suggest that the development of assays for mutagenesis and carcinogenesis with toadfish cells be based upon primary cultures as are a number of assays for cell transformation and unscheduled DNA synthesis that utilize mammalian material. The use of primary cultures to assay chemicals for genotoxic activity confers certain advantages. Primary cultures appear to retain a greater capacity for xenobiotic metabolism than do cells which have been in culture for a longer period of time.

## DISCUSSION

This series of preliminary studies collectively suggest that the oyster toadfish is a suitable organism with which to pursue investigations of the

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Figure 26. The effects of no treatment and pretreatment of fish and rats with a 3-methylcholanthrene (MC) and Aroclor 1254 (AC) on the activation of benzo (a) pyrene (BP) and 2-aminoanthracene (AA) to Salmonella (TA98) mutagens.
Four graphs: a. Rat S-9, BP; b. rat S-9, AA; c. fish S-9, BP; d. Fish S-9, AA.

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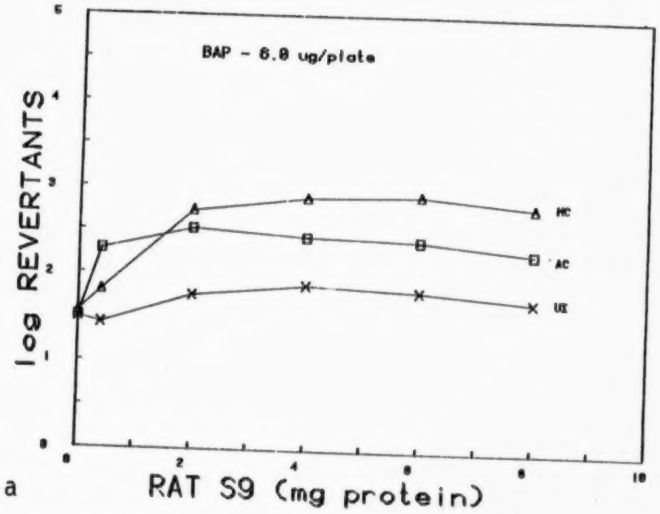
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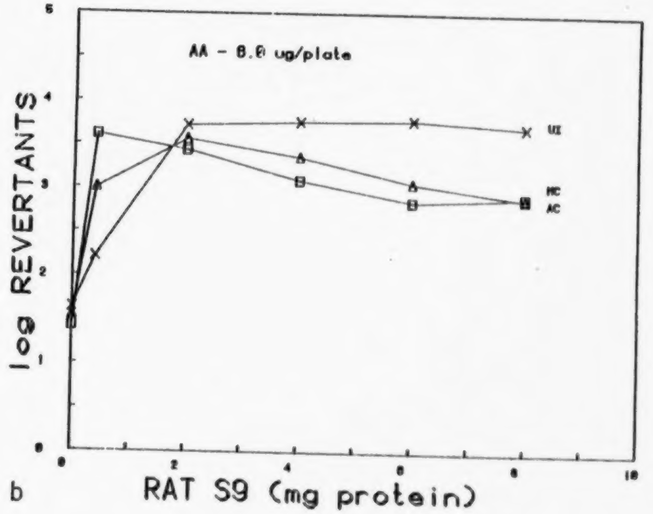
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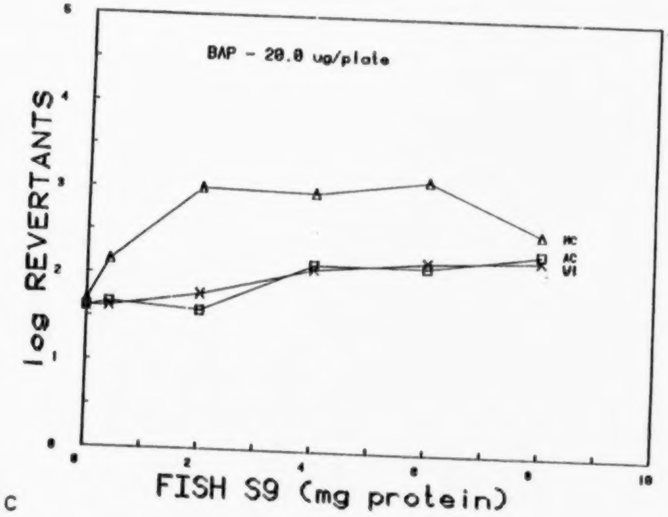
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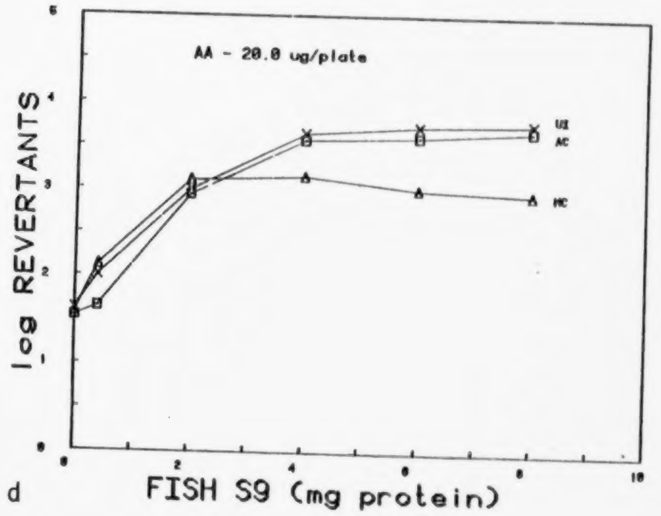
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d

Table 28. Culture of Tissues from *Rivulus marmoratus* and *Opsanus tau*

| Species | Tissue | Method[a] | Number Cultures Initiated | Number Cultures Growing | Number Successful Subcultures | Maximum Number Subcultures |
|---|---|---|---|---|---|---|
| *Rivulus marmoratus* | liver | E, T | 6 | 1 | 0 | 0 |
| | gonad | E, T | 2 | 0 | 0 | 0 |
| | fin | E | 2 | 0 | 0 | 0 |
| *Opsanus tau* | liver | E, T | 37 | 15 | 1 | 1 |
| | ovary | E, T | 26 | 18 | 5 | 4 |
| | skin | E, T | 25 | 3 | 0 | 0 |
| | spleen | E, T | 9 | 1 | 0 | 0 |
| | other | E, T | 28 | 1 | 0 | 0 |

[a] E = explant; T = trypsin dispersion

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effects of mutagens and carcinogens on marine teleost fish. Furthermore, the toadfish is a suitable organism to use as a model for evaluating the use of cytogenetic effects on the detection, identification and assessment of hazards of water borne chemicals in estuarine ecosystems. The following summarizes the features that recommend the toadfish for further investigation:

1) The toadfish has a karyotype suitable for cytogenetic analysis.

2) Leukocytes obtained from toadfish can be cultured and provide a system suitable for SCE analysis following in vitro exposure to mutagenic chemicals (Maddock and Kelly, 1980).

3) The toadfish is sufficiently large to provide gram quantities of tissue needed for metabolic evaluation for primary culture initiation and for pharmacokinetic investigation.

4) The toadfish is tolerant of handling out of water for extended periods and will survive adverse conditions that are not tolerated by many other species.

5) Toadfish are available in abundance from Massachusetts to Florida and are available to any laboratory on the Atlantic coast.

6) The toadfish is a sedentary, bottom-dweller and is associated with the sediments where many potentially toxic chemicals tend to accumulate.

7) The capacity for xenobiotic metabolism in toadfish is similar to that of mammals.

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